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In order to preserve continuity the present sequence of volume numbers will be retained, and in each case the volume for 1951 will be Volume 29.

The subscription rates for the Journals will remain as at present.

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ANTIBIOTICS PRODUCED BY MICROCOCCI AND STREPTOCOCCI THAT SHOW SELECTIVE INHIBITION WITHIN THE GENUS *STREPTOCOCCUS*[1]

BY R. G. E. MURRAY[2] AND L. J. LOEB

Abstract

Two strains of *Micrococcus epidermidis* and five of *Micrococcus pyogenes* were found to produce antibiotics that were detected by using a mucoid *Streptococcus pyogenes* as test organism. The antibiotics were also active against some other Gram-positive organisms, but not against Gram-negative bacteria. The *M. epidermidis* strains inhibited most β-haemolytic streptococci with the exception of mucoid Lancefield Group C. The *M. pyogenes* strains were active against the majority of mucoid *S. pyogenes* (Group A), but few or none of the nonmucoid strains were inhibited. Streptococci of Groups B, C, and G were not affected. On the basis of antibacterial spectrum and characteristics of the antibiotics (dialysis, stability, and production curves) the seven strains were divided into four groups producing antibiotics of different types.

Three strains of β-haemolytic streptococci were tested which were known to produce antibiotics. Two of these showed a selective inhibition similar to the micrococci. They inhibited all of the mucoid *S. pyogenes* but few of the nonmucoid; they inhibited all strains of Group C, both mucoid and nonmucoid, but did not inhibit the Group G strains tested.

These observations suggest that, apart from capsulation, there may be a fundamental metabolic difference between the majority of mucoid and nonmucoid strains of *S. pyogenes*.

Introduction

While testing strains of *Micrococcus pyogenes* for the production of hyaluronidase by a streak-plate method, Murray and Pearce (15) noted that a small number of strains inhibited the mucoid *Streptococcus pyogenes* used as indicator organism. To determine whether this interference could be avoided, other mucoid *S. pyogenes* were tried and most of these were antagonized by the inhibitory micrococci. It was also noted that stock nonmucoid strains were not inhibited. This preliminary observation led to a more detailed study of the inhibitory strains. The possibility was kept in mind that selective antibiotics might direct attention to metabolic systems that distinguish closely related streptococci.

A similar situation arose when Sherwood (20) was testing β-haemolytic streptococci for hyaluronidase production by the same technique. A number of strains were found, representing nine Lancefield Groups, that inhibited the

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ont.

[2] *Aided by a grant from the National Research Council, Canada.*

test organism and certain other streptococci. Three of these strains, kindly provided by Dr. Sherwood, have been included in this study because of the similarity of the method of selection.

Methods

The micrococci were surveyed by a streak method on blood agar plates. The test organism was the mucoid strain of S. *pyogenes* (M. Strep. 9) used by Murray and Pearce (15). Active *Micrococcus* strains and the *Streptococcus* strains obtained from Dr. Sherwood were tested in the same way against a variety of organisms to determine their antibacterial spectra. The plates were incubated aerobically. During the course of the work the effect of incubation anaerobically or under increased carbon dioxide tension was investigated. (There was no change in the results except that one *Micrococcus* (3064) was not active in the carbon dioxide atmosphere.) This testing procedure on blood agar largely excluded the possibility that activity might be due to peroxides.

A cell-free fluid containing the antibiotic was obtained from all but one of the active strains by a variation of a "cellophane sac" technique described by Heatly and Florey (9). Cellophane tubing was suspended from a plugged tube in the stopper of a side arm vacuum flask and autoclaved. This sac was filled with sterile broth and the inoculum was distributed over the outer surface of the cellophane *via* the side arm of the flask. The apparatus was incubated at 37° C. and the inhibitory action of the broth was tested at intervals during a 14 day period.

Activity was determined qualitatively by using standard glass cylinders or filter paper discs $\left(\frac{1}{2}\text{ in.}\right)$ on blood agar plates seeded with the test organism. Quantitative estimations were made by serial halving dilutions in dextrose broth for a rough estimate, and by harmonic dilutions for a more accurate titration. An inoculum of 0.1 ml. of a 24-hr. dextrose-broth culture of the test organism in a total volume of 5 ml. was used for each dilution. Each series was incubated for 18 to 24 hr. at 37° C. The end point of a titration was read as the highest dilution of antibiotic showing complete absence of growth.

Media

The *blood agar* used for the survey was that used by Murray and Pearce (15). The *broth* used for antibiotic production in the cellophane sac consisted of:

Ficin digest of meat*	400 ml.
Proteose peptone #2 (Difco)	5 gm.
Proteose peptone #3 (Difco)	5 gm.
Distilled water to 1 liter	pH 7.0

* *Similar to the papain digest medium of Asheshov (1) but substituting ficin for papain.*

The *dextrose broth* used for titration of activity consisted of:

Nutrient broth, dehydrated (Difco)	8 gm.
Proteose peptone #2 (Difco)	5 gm.
Proteose peptone #3 (Difco)	5 gm.
Dextrose	12 gm.
Distilled water to 1 liter	pH 7.4

Results

Micrococcal Antibiotics

The Incidence of Inhibitory Micrococci

Five of 158 coagulase-positive micrococci and two of 47 coagulase-negative micrococci showed antibiotic activity against M. Strep. 9. The five inhibitory *M. pyogenes* strains (1831, 1648, 4457, L8400, 3064) were isolated from human secretions. One of the coagulase-negative organisms (2921) originated from a sample of cream pie while the other (CI) was isolated as a plate contaminant; they were probably derived from human sources and they correspond closely to *M. epidermidis*.

Range of Activity of Inhibitory Micrococci

Active strains found in the initial screening were further tested, by the streak method, against a variety of organisms. The effect on various species of *Streptococcus* is summarized in Table I, and on some other Gram-positive

TABLE I

Susceptibility of strains of *Streptococcus* to inhibitory micrococci

Microorganisms surveyed	No. of strains tested	Number of strains inhibited by:					
		1831	1648	4457	L8400	3064	2921 or CI
Beta-haemolytic streptococci							
Lancefield Group A nonmucoid	23	1	2	2	0	0	22
Lancefield Group A mucoid	8	6	7	7	6	3	8
Lancefield Group B nonmucoid	6	0	0	0	0	0	4
Lancefield Group C nonmucoid	6	0	0	0	0	0	5
Lancefield Group C mucoid	6	0	0	0	0	0	0
Lancefield Group G nonmucoid	6	0	0	0	0	0	6
Alpha-haemolytic (*viridans*) streptococci	8	2	3	3	2	3	3

organisms in Table II. The two *M. epidermidis* strains gave identical results. None of the active strains inhibited any of the Gram-negative bacteria that were tested: *Escherichia coli*, *Salmonella typhosa*, *Salmonella schottmuelleri*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Proteus* sp., *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Neisseria catarrhalis*.

TABLE II

SUSCEPTIBILITY OF VARIOUS GRAM-POSITIVE ORGANISMS TO INHIBITORY MICROCOCCI

Microorganisms surveyed	No. of strains tested	Number of strains inhibited by:					
		1831	1648	4457	L8400	3064	2921 or CI
Micrococcus pyogenes	13	4	11	11	0	7	6
Micrococcus sp. coagulase-negative	4	1	1	1	0	0	1
Diplococcus pneumoniae (various types)	9	9	9	9	2	1	7
Corynebacterium diphtheriae	6	6	6	6	0	6	0
Bacillus sp.	4	2	3	3	0	4	0

The range of activity shown in the streak tests allowed preliminary classification of the antibiotics produced. The activity of the *M. epidermidis* strains was clearly differentiated from the others and showed a more general activity against the streptococci and no action against *Corynebacterium diphtheriae*. Three of the *M. pyogenes* strains (1831, 1648, and 4457) seemed to be almost identical; strain 3064, although somewhat resembling these, was later differentiated by another property (see Table III). L8400 was distinct owing to its narrow range of activity.

The selective inhibition of certain streptococci is illustrated in Table I. The *M. pyogenes* strains were active against few or none of the nonmucoid Lancefield Group A streptococci, whereas they inhibited most of the mucoid strains of the same group. On the other hand these strains inhibited none of the streptococci of Lancefield Groups B, C, and G. The *M. epidermidis* strains inhibited the majority of the streptococci tested, excepting mucoid Group C.

In addition to M. Strep. 9 another strain of Group A type 9 mucoid *S. pyogenes* and seven nonmucoid type 9 strains were available. The mucoid strains were sensitive to all of the inhibitory *M. pyogenes* except 3064, while none of the nonmucoid strains were sensitive. All were inhibited by *M. epidermidis* 2921. A similar result was obtained with a series of Group A type 6 cultures.

Attempts to obtain stable mucoid variants of the nonmucoid Lancefield Group A *S. pyogenes* strains were unsuccessful, but on two occasions nonmucoid variants of M. Strep. 9 were obtained. Both of these variants were inhibited by the same strains of *Micrococcus* and to the same degree as the parent strain.

Characteristics of the Inhibitory Substances

All the inhibitory strains except 3064 produced inhibitory substances that diffused through cellophane. This was tested by growing the micrococci on the surface of a piece of cellophane placed on a blood agar plate. The cellophane and growth was removed after overnight incubation and the area so

exposed was seeded with a sensitive organism and incubated to find out whether diffusion had taken place. The cellophane sac technique for obtaining active, cell-free broth could be applied to the organisms producing dialyzable antibiotics. The broth inside the sacs was tested daily by the assay cylinder method and halving dilution titration. Strain 2921 showed a steady production of antibiotic up to the eighth day (Fig. 1); the activity of this broth has

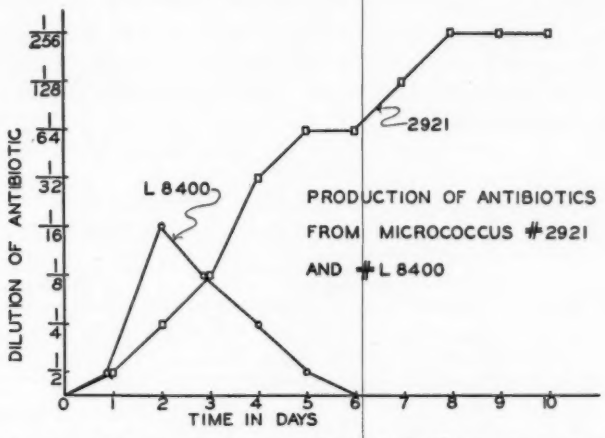

FIG. 1. *A graph showing the titers of antibiotic obtained during production in cellophane sacs using strains 2921 and L8400.*

remained constant for 18 months at 4° C. Strains 1831, 1648, 4457, and L8400 showed greatest inhibition at two – five days, after which the activity declined (Figs. 1 and 2). L8400 had an earlier peak. No satisfactory means was found for producing the antibiotic from 3064.

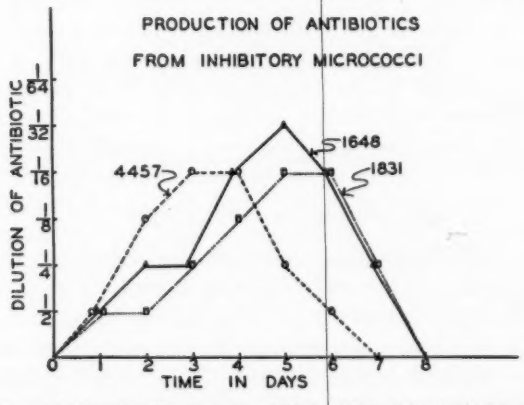

FIG. 2. *A graph showing the titers of antibiotic obtained during production in cellophane sacs using strains 4457, 1648, and 1831.*

Bacteriostatic or bactericidal action was determined in the tubes of an antibiotic titration. Tubes showing inhibition after overnight incubation were diluted with broth and reincubated to detect growth. The mode of action of the inhibitory micrococci is bacteriostatic, although two times the minimum inhibitory dose of the antibiotic produced by *Micrococcus* 2921 seemed to have bactericidal action towards M. Strep. 9. The action of 3064 is unknown.

Table III summarizes the characteristics of the inhibitory micrococci. The organisms are arranged in four groups on the basis of the coagulase test and properties of the antibiotic substances. The antibacterial spectra gave the same general classification.

TABLE III

SOME CHARACTERISTICS OF ANTIBIOTIC-PRODUCING MICROCOCCI

	Coagulase-positive strains: *M. pyogenes*		Coagulase-negative strains: *M. epidermidis*	
	Group			
	I	II	III	IV
Strains	1831 1648 4457	L8400	3064	2921 CI
Incidence	$\frac{3}{158}$	$\frac{1}{158}$	$\frac{1}{158}$	$\frac{2}{47}$
Hyaluronidase	+	−	+	−
Pigment	Golden	Faint golden	Golden	White
Antibiotic characteristics				
Dialysis	+	+	−	+
Stability	−	−	−	+
Peak of production in days	4 – 5	2	?	8

STREPTOCOCCAL ANTIBIOTICS

As has already been mentioned, Sherwood *et al.* (20) isolated a number of streptococci that inhibited other *Streptococcus* strains. They used the same test organism (M. Strep. 9) that was used in this study.

Three active strains (one representative from each of Lancefield Groups A, D, and H) were obtained from Sherwood. Their activity against selected organisms is shown in Table IV. The streptococcal and micrococcal antibiotics were similar in the overall spectrum of activity. Two strains (Groups

TABLE IV

SUSCEPTIBILITY OF VARIOUS GRAM-POSITIVE ORGANISMS TO INHIBITORY STREPTOCOCCI

Organisms surveyed	No. of strains tested	Number of strains inhibited by:		
		C203 Lance. A	AND Lance. D	"H" Lance. H
Streptococcus				
Lancefield Group A, mucoid	9	9	9	9
Lancefield Group A, nonmucoid	21	3	3	21
Lancefield Group B, nonmucoid	1	1	1	1
Lancefield Group C, mucoid	6	6	6	6
Lancefield Group C, nonmucoid	4	4	4	4
Lancefield Group G, nonmucoid	5	0	0	3
Micrococcus pyogenes	10	0	0	0
Corynebacterium diphtheriae	5	0	0	5

A and D) inhibited all the mucoid Group A strains and relatively few of the nonmucoid strains, while the Lancefield Group H strain showed a more general inhibition. The three streptococci inhibited all the Group C strains. Data for *M. pyogenes* and *C. diphtheriae* are provided for comparison with the antagonistic micrococci.

Discussion

The production of inhibitory substances by *M. pyogenes* and other micrococci is not a new observation. De Freudenreich (7), Doehle (3), and Löde (13) described the inhibitory action of occasional strains. Schiotz (17) used a strain of *M. pyogenes* for the treatment of diphtheria carriers, an application that was revived by Dulescouet and Ballet (6) and by Lesbre and Merle (12). Dujardin-Beaumetz (4) isolated an *M. pyogenes* active against Gram-positive organisms and an unidentified *Micrococcus* (5) that inhibited the growth of a very wide range of bacteria including Gram-negative organisms. Prica (16) reported a strain of *M. pyogenes* active against species of *Klebsiella*. The above studies give little information other than the range of species inhibited as a basis for comparison. A few recent studies are more detailed. Jennings and Sharp (10) reported the isolation of a number of strains of *M. pyogenes* whose antibacterial spectrum was confined to Gram-positive bacteria. One of these organisms was investigated in detail by Gardner (8) who isolated a nondialyzable antibiotic and described its characteristics. Su (21) has also given a detailed description of "micrococcin", an antibiotic produced by a *Micrococcus* isolated from sewage. Most of the inhibitory strains are active against various Gram-positive and inactive against Gram-negative organisms as are those reported here. Two exceptions are noted in the strains described by Dujardin-Beaumetz (5) and by Prica (16); the former inhibited a notably wide range of Gram-negative organisms.

Two of the four groups (Table III) of antagonistic micrococci we have isolated show similarities to some of the strains reported in the literature. The three strains of Group I correspond to the *M. pyogenes* isolated by Dulescouet and Ballet (6) and Lesbre and Merle (12). The *Micrococcus* 3064 (Group III) is similar to the *M. pyogenes* strains described by Dujardin-Beaumetz (4) and by Gardner (8). The antibiotics of Groups II and IV do not seem to have counterparts reported in the literature.

The *M. epidermidis* strains inhibited the majority of β-haemolytic streptococci tested, but did not inhibit the mucoid Group C strains. The *M. pyogenes* strains, differing in their specificity, were active against a high proportion of mucoid Group A streptococci, a very few nonmucoid Group A and not against any representatives of Groups B, C, or G. This indicates not only some specificity as to group, but also selection of a distinct morphological variant within the group. For all these antibiotics, it is the susceptibility of the mucoid variation that is distinguished. In one case, mucoid Group A are largely susceptible; in the other case, mucoid Group C are notably resistant. Since the two mucoid organisms have a hyaluronic acid capsule in common (11, 14, 18, 19), it is tempting to speculate that the antibiosis is dependent upon some metabolic system associated with capsulation. However, with each antibiotic, if either one of the mucoid groups is inhibited the other is not. Therefore, the apparent association of susceptibility with the mucoid state and the hyaluronic acid capsule is probably fortuitous.

The antibiotic-producing streptococci discovered by Sherwood (20) resemble the micrococci in that their activity is confined to Gram-positive organisms. Two of the three strains of inhibitory streptococci that were tested also showed a selective inhibition, within Group A, of the mucoid strains (Table IV). In fact, these are rather more selective than were the micrococci since all the mucoid strains were inhibited. A further contrast was that both mucoid and nonmucoid Group C strains were inhibited.

It is generally believed that most antibiotics owe their activity to competitive inhibition of enzyme systems. Assuming that the selective antibiotics described in this paper act in this fashion, it is possible that the specificities observed may indicate variations in physiological mechanisms within the β-haemolytic streptococci. These streptococci exhibit considerable variations in pathogenicity and host specificity that correlate well with the immunological grouping. If the assumption is true that the antibiotics act upon specific enzyme systems, the selection of mucoid Group A strains may be interpreted in two ways: (1) The mucoid mutation may involve some more fundamental enzyme system than that needed for capsule production, or (2) the mucoid mutation is most often associated with one physiological kind of *S. pyogenes*, which may be independent of immunological type. Of these two hypotheses, the latter fits the facts more closely. Most of the mucoid strains but only a few of the nonmucoid strains were susceptible. Further, nonmucoid mutants of a susceptible mucoid strain had the same sensitivity as the parent. A similar sort of experimental approach is not possible with

our Group C representatives, because the mucoid strains are *Streptococcus zooepidemicus* ("animal pyogenes") and the nonmucoid are *Streptococcus equisimilis* ("human C"), which have other distinguishing properties (2).

Such arguments lend color to the possibility that selective antibiotics may direct attention to new and worthwhile attacks upon the physiology of the β-haemolytic streptococci and *S. pyogenes* in particular.

Another aspect of the inhibitory micrococci may be the role they could play in the ecology of skin and mucous membranes. This activity could occur only in occasional situations, since active strains form only about three per cent of isolates.

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THE CHEMICAL ANALYSIS OF PURIFIED INFLUENZA VIRUS A (PR8 STRAIN) CONTAINING RADIOACTIVE PHOSPHORUS[1]

By A. F. Graham

Abstract

Purified influenza virus A (PR8 strain) was found to contain about 11% phospholipid and 5% nucleic acid in agreement with previously reported work. The method of Schmidt and Thannhauser, applied to the nucleic acid fraction of the virus, indicated the presence of 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. When influenza virus was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphorus both phospholipid and nucleic acid components of the virus were found to contain the isotope. The specific radioactivity of the nucleic acid fraction was about four times that of the phospholipid.

Introduction

In previous work (4, 5) it was shown that when the PR8 strain of influenza virus A was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphate, the virus, when subsequently purified, contained a small amount of the isotope. There was no exchange of P^{32} between the virus and inorganic phosphate and it was concluded that the virus had been labelled with the isotope during its growth in the cells.

Since it has been reported by Taylor (15) that the greater part of the phosphorus of the virus is contained in the phospholipid and nucleic acid it was of interest to determine whether the P^{32} in the radioactive virus was also present in these components. If either or both of the phospholipid and nucleic acid were found to be labelled, it would afford additional evidence that the isotope was incorporated into the structure of the virus. The work described in this paper indicates that both phospholipid and nucleic acid were labelled.

Analyses of the nucleic acid content of influenza virus, using quantitive colorimetric tests for pentose and desoxypentose, have been reported by Taylor (15) and by Knight (7). While both pentose and desoxypentose nucleic acids seem to be present, there is still some doubt over their exact proportions, as pointed out by Beard (1). The present work indicates that these colorimetric tests for nucleic acids may be subject to interference from other constituents present in the virus particle and consequently an independent method, that of Schmidt and Thannhauser (11), has been applied in an attempt to determine the relative proportions of the two nucleic acids. From the results of these analyses it would appear that the PR8 strain of influenza virus A contains about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid.

[1] *Manuscript received June 6, 1950.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. Aided by a grant from the National Cancer Institute of Canada.

Methods

Determination of Total Phosphorus

The method has been fully described in a previous paper (5).

Determination of Radioactive Phosphorus

All the radioactivity measurements were made on solutions of material in water or organic solvents. The method, which was also used throughout the previously reported work (4, 5), is described here in detail.

The radioactive solutions were pipetted onto $1\frac{1}{8}$ in. diameter filter paper discs fixed to the tacky surface of a 2 in. wide strip of "Scotch" cellophane tape. After drying the discs under an infrared lamp a second strip of tape was pressed firmly over the top. An aliquot of 0.2 ml. of solution was sufficient to saturate the paper discs; where the radioactivity of the material was low, several 0.2 ml. aliquots were added to each disc, the paper being dried between additions. The cellophane tape was trimmed off close to the edge of the disc, leaving a "sandwich" of filter paper between two layers of tape. This was fixed in a brass mount and inserted under a Geiger-Müller counter tube of the end-window type. The brass mount was constructed in such a way that the radiation from an area of the filter paper exactly 1 in. in diameter registered on the counter.

A routine set of five radioactivity determinations was made with this method on an aqueous solution of P^{32}, as inorganic phosphate, to determine its accuracy. With an average counting rate of 3450 impulses per min. (150 times background) the standard error of the mean was 1.1%. Inverting the paper discs under the counter had no measurable effect on the counting rate, nor did a total of three thicknesses of cellophane tape over the discs reduce the number of impulses registered.

As the method was used for estimating the radioactivity in biological fluids which contained appreciable amounts of protein, it was necessary to determine whether the presence of protein would interfere with the determination. Accordingly, a series of twofold dilutions of a solution of P^{32}, as inorganic phosphate, was made in 2% haemoglobin. At the same time, a similar series of dilutions of the same solution was made in distilled water. Radioactivity determinations were made on each series of dilutions as described above. In both series, the activity decreased in direct ratio to the concentration of P^{32}, the activities of corresponding dilutions in each series being the same within experimental error.

The method was calibrated against aqueous solutions of two uranium salts (C.P. grade) both of which had stood at least two years to ensure that they were in equilibrium. The β-ray disintegration rates of the uranium sources were calculated from data given by Kamen (6). This calibration was later checked against a RaD + E standard source from the National Bureau of Standards, Washington. A solution of P^{32} calibrated against the Washington

standard contained 5330 μrd. per ml. When measured against a uranium acetate solution it contained 5880 μrd. per ml. and against a uranium nitrate solution 5950 μrd. per ml.

In this paper, as in the previous report (5), the activities of P^{32} sources are expressed in counts per minute (c.p.m.); the results may be converted to microrutherfords using the factor 1000 c.p.m. as equivalent to a β-ray activity of 85.8 μrd.

Determination of Pentose Nucleic Acid (PNA) by the Orcinol Method

PNA was estimated by the orcinol method according to the directions of McRary and Slattery (10). Two specimens of commercial yeast nucleic acid containing 7.90% P (Schwartz) and 7.85% P (Eimer and Amend) were used as standards. Although the green color developed in this test is due to the pentose in PNA the results were expressed in terms of the phosphorus content of the nucleic acid, that is, as pentose nucleic acid phosphorus (PNAP). When per cent transmittance, read at 660 mμ against reagent blanks, was plotted against PNAP for the two PNA specimens the curves were linear between 0-13 μgm. PNAP and superimposable.

According to Schneider (12, 13) the nucleic acids of animal tissue may be completely extracted with 5% trichloroacetic acid (TCA) at 95° C. This method was applied to one of the specimens of PNA (Schwartz). The nucleic acid was extracted completely in 15 min., judged by phosphorus analysis, leaving a small residue presumably of protein. When the orcinol reaction was applied to a series of dilutions of the extract and the results plotted in terms of phosphorus content, the curve was identical with the two former curves.

Since the slopes of standard curves obtained in this test varied from one set of determinations to another owing to small variations in conditions of color development, a set of five dilutions of one of the standard PNA solutions was always run at the same time as an unknown solution. Thus the color density of the unknown solution could always be referred to a standard curve obtained under exactly the same conditions.

Determination of Desoxypentose Nucleic Acid (DNA) by the Diphenylamine Reaction

The diphenylamine reaction for DNA was carried out according to the directions of Seibert (14). Two specimens of calf thymus nucleic acid containing 7.32% P and 8.20% P were used as standards.* When per cent transmittance at 600 mμ of the color developed in the diphenylamine reaction was plotted against phosphorus content for each of the two specimens, the two curves were superimposable and linear between 0-50 μgm. DNAP. A weighed quantity of one of the specimens was extracted with 5% TCA at 95° C. The extract contained all the phosphorus of the original DNA and in the diphenylamine test gave a curve almost identical to the two former curves.

* *I am indebted to Dr. G. C. Butler for one of the specimens of calf thymus nucleic acid.*

As in the orcinol test a set of five dilutions of a standard DNA solution was always run simultaneously with an unknown solution.

Preparation of Purified Influenza Virus

Purified influenza virus was prepared by the method which has already been fully described (5).

Experimental

Chemical Analysis of Influenza Virus

Before carrying out any analytical work on radioactive virus, it was necessary to determine the distribution of phosphorus between the virus constituents. For analysis, suspensions of purified influenza virus were prepared. An aliquot was taken for total phosphorus estimation and a further aliquot for dialysis against several changes of distilled water at 5° C. After 48 hr. the agglutinated virus was sedimented, dried *in vacuo* from the frozen state and then over phosphorus pentoxide in a vacuum desiccator.

The weighed dry material (10-25 mgm.) was stirred mechanically for 45 min. at room temperature with 4 ml. of alcohol–ether mixture (3/1, v./v.). The extraction was repeated on the sediment after centrifugation and the material was finally washed once with a small amount of ether and dried *in vacuo*. This alcohol–ether insoluble residue was considered to contain the protein and nucleic acid of the virus.

The combined alcohol–ether extracts were evaporated to dryness at 30° C. in a stream of nitrogen and the residue was extracted with several small portions of petroleum ether (b.p. 40°-60° C.) at room temperature. In accordance with the earlier work of Taylor (15) this extract was assumed to contain the phospholipid of the virus. Phosphorus and weight determinations were made on the various fractions.

The results of the fractionation are shown in Table I, the figures representing analyses on four different virus preparations. In each row of the table, the figures are averages of results obtained on at least three of the four preparations since all the determinations were not carried out on every preparation.

TABLE I

CHEMICAL ANALYSIS OF INFLUENZA VIRUS A (PR8 STRAIN)

Fraction	Percentage of virus weight*	Phosphorus, % of virus weight*
Dried whole virus	100	0.96
Alcohol–ether soluble	26	0.51
Petroleum ether soluble	24	0.48
Petroleum ether insoluble	—	0.04
Alcohol–ether insoluble	76	0.45

* *Values are based on dry weight of virus.*

The virus would appear to contain about 11% phospholipid, calculated from the phosphorus content of the petroleum ether soluble fraction. The results are in agreement with those of Taylor (15) for this virus.

Fractionation of the Alcohol–Ether Insoluble Residue

A strongly positive orcinol test for pentose was obtained with the alcohol–ether insoluble residue. While an unmistakably positive diphenylamine test for desoxypentose was not obtained on this fraction nor on whole virus, in our hands this has proved to be a relatively insensitive color reaction. Moreover the diphenylamine reaction is subject to interference by proteins and other substances as indicated by von Euler and Hahn (17).

Therefore, an attempt was made to separate and estimate the two nucleic acids of the virus by the method of Schmidt and Thannhauser (11). This procedure was tested out on a mixture of calf thymus nucleic acid, yeast nucleic acid, and crystalline egg albumen of known phosphorus contents, the recovery of the two nucleic acids being almost quantitative when present in roughly equal proportions. When the method was applied to the alcohol–ether insoluble residues of four different preparations of influenza virus 94.0%, 90.6%, 93.7%, and 93.5% of the total phosphorus of the residue appeared with the PNA fraction.

If phosphoprotein were present in the virus, its phosphorus would appear in the PNA fraction as inorganic phosphate. The procedure of Delory (3) was therefore applied to the separated PNA fraction, in two cases, to precipitate any inorganic phosphorus so that it could be estimated separately. In neither case was more than a trace of inorganic phosphorus found. It is well to mention, however, that the precipitation of inorganic phosphate was not quantitative when the amount of inorganic phosphorus present was less than 15 μgm. per ml. To avoid this difficulty the precipitation method of Delory was applied to solutions containing known amounts of inorganic phosphate (0-30 μgm. P per ml.) and a calibration curve prepared relating inorganic P added to that recovered from the precipitation. When a solution was made up containing 8.70 μgm. P per ml. as inorganic phosphate and 5.55 μgm. P per ml. as yeast nucleic acid phosphorus, using the precipitation method and calibration curve, the inorganic phosphate was estimated as 8.40 μgm. P per ml.; the precipitation was carried out under the same conditions as obtained in the influenza virus experiments.

It would appear from these results that about 6% of the phosphorus of the alcohol–ether insoluble residue of the virus is present in DNA and about 94% in PNA. This would be equivalent to approximately 0.3% DNA and 4.5% PNA in the whole virus.

Determination of PNA and DNA in the Virus by Colorimetric Tests

Since the above results for PNA and DNA in the virus were quite different from previously reported results (7, 15) an attempt was made to determine the two nucleic acids by quantitative colorimetric tests.

Following separation of the two fractions from the alcohol–ether insoluble residue of the virus by the Schmidt and Thannhauser method, both orcinol and diphenylamine tests were carried out on the PNA fraction. It was observed that practically all the phosphorus could be extracted from the DNA fraction with 5% trichloroacetic acid for 30 min. at 90° C.; the diphenylamine test for DNA was carried out on this extract. The results are recorded in Table II. Since the orcinol and diphenylamine reactions were standardized

TABLE II

DISTRIBUTION OF PHOSPHORUS IN COMPONENTS OF ALCOHOL-ETHER INSOLUBLE RESIDUE OF INFLUENZA VIRUS SEPARATED BY SCHMIDT AND THANNHAUSER METHOD

Fraction	Total phosphorus, μgm.	Phosphorus by orcinol test, μgm.	Phosphorus by diphenylamine test, μgm.
Pentose nucleic acid	62.8	53.8	7.7
Desoxypentose nucleic acid	4.3	—	2.6

in terms of the phosphorus contents of the respective nucleic acids as explained earlier, the figures in columns three and four of Table II give the amounts of phosphorus associated with these two acids in the virus. It is observed that the total amount of phosphorus determined from the colorimetric tests in this experiment checks fairly well with the total phosphorus estimated by direct determination. The PNA fraction separated by the Schmidt and Thannhauser method would appear to contain a small amount of DNA judged by the results of the diphenylamine test. The orcinol reaction was carried out on the DNA fraction but it was impossible to determine whether or not a slight green color had formed because of the presence of a brown pigment which appeared during color development. Similar results were obtained with a second preparation of virus.

It should be remarked that despite the reasonable agreement between the results of the two methods as shown in Table II, the colorimetric estimations came under suspicion for two reasons. Firstly, both ribose and yeast nucleic acid yielded a clear brilliant green color in the orcinol reaction whereas the influenza virus fractions often gave an olive green color with a tendency to slight opalescence. Secondly, the diphenylamine tests on the virus fractions were almost invariably slightly brown in color instead of the pure blue obtained with DNA in this reaction. Both these observations suggested the presence of substances in the virus which might interfere in the color tests.

Evidence that the colorimetric tests were not entirely trustworthy when applied to influenza virus fractions was obtained in a further experiment. The alcohol–ether insoluble residue from a purified virus preparation was treated for 30 min. with 5% trichloroacetic acid at 90° C. in an attempt to extract completely the nucleic acids. Eighty-eight per cent of the phosphorus

was removed by this treatment, 87% in a second such experiment. Both orcinol and diphenylamine tests were applied to the extract and to the extracted residue. The results are shown in Table III. It is observed that

TABLE III

DISTRIBUTION OF PHOSPHORUS IN HOT TRICHLOROACETIC ACID EXTRACT AND RESIDUE OF ALCOHOL–ETHER INSOLUBLE PORTION OF INFLUENZA VIRUS

Fraction	Total P, μgm.	P by orcinol test, μgm.	P by diphenylamine test, μgm.
Trichloroacetic acid extract	167.3	157	41.8
Extracted residue	23.3	37.5	12.0

the total amount of phosphorus in the alcohol–ether insoluble fraction of the virus estimated by the colorimetric tests, was about 30% greater than the amount of phosphorus found to be present by the direct estimation of total phosphorus.

Application of the orcinol reaction of Tillmans and Phillipi (16), standardized against glucose, to the trichloroacetic acid extract mentioned above indicated a carbohydrate content of about 7% of the weight of the virus. Estimations of carbohydrate by this method were not done on the trichloroacetic acid extracted residue.

Chemical Fractionation of Radioactive Influenza Virus

In order to determine the distribution of P^{32} between the phosphorus containing constituents of the labelled virus, two samples of purified radioactive virus were prepared from two groups of eggs which for Preparation I received 56,700 c.p.m. of P^{32} in each egg, and for Preparation II, 65,000 c.p.m. in each egg. Following the adsorption and elution technique with red cells previously described, the virus was subjected to two cycles of differential centrifugation, the virus being resuspended each time in 0.85% (w./v.) sodium chloride solution.

A known volume of Preparation I was then dried *in vacuo* from the frozen state, while an aliquot of Preparation II was agglutinated by dialysis against distilled water before drying in the same manner. Both dried preparations were then fractionated by the same methods as described for the nonradioactive virus. Total phosphorus and P^{32} estimations were carried out on each fraction; the specific activities were calculated and are shown in Table IV along with the results for total phosphorus. The total phosphorus figures for whole virus represent the total amounts of phosphorus in the aliquots taken for drying and for dialysis in Preparations I and II respectively. Phosphorus and radioactivity estimations on the alcohol–ether insoluble fractions were

TABLE IV

SPECIFIC ACTIVITIES OF FRACTIONS OF INFLUENZA VIRUS LABELLED WITH P^{32}

Fraction	Preparation I		Preparation II	
	Total phosphorus, μgm.	Specific activity, c.p.m./μgm. P	Total phosphorus, μgm.	Specific activity, c.p.m./μgm. P
Whole virus	94.5	6.6	113.6	8.8
Petroleum ether soluble	46.0	3.0	17.9	3.6
Petroleum ether insoluble	1.5	Trace	33.5	3.6
Alcohol–ether insoluble	46.5	10.8	44.7	16.5

carried out on solutions of the material in 1.0 *N* potassium hydroxide, blank corrections being made for the very small natural radioactivity of potassium.

For Preparation II, the sum of the phosphorus figures for the three fractions is only 85% of the phosphorus in the whole virus. This difference could be accounted for by loss of virus in the manipulations attendant upon dialysis. Further, in this preparation the extraction of phospholipid from the alcohol–ether soluble fraction with petroleum ether seems to have been incomplete, judged by the amount of phosphorus containing material remaining in the petroleum ether insoluble fraction. The two experiments, however, are consistent in that they indicate the specific activity of the nucleic acid portion of the virus to have been 3.5 – 4.5 times that of the phospholipid.

In Preparation I, an attempt was made to purify the phospholipid by precipitation with magnesium chloride and acetone according to the method of Bloor (2) to find whether the specific activity would be changed by this treatment. After purification there was insufficient material left to allow an accurate radioactivity estimation. An attempt was also made to separate the PNA and DNA by the method of Schmidt and Thannhauser as described in a previous section. By far the largest proportion of the radioactivity was associated with the PNA fraction; it was impossible to get an accurate estimate of the small amount of activity remaining with the DNA.

Discussion

As far as the chemical analysis of purified influenza virus has been carried in the present work the results are in agreement with previous work, except for the relative amounts of pentose and desoxypentose nucleic acids. On the assumption that the phosphorus of the virus was contained only in phospholipid and nucleic acid, the total amount of nucleic acid was found to be about 5%, calculated from the phosphorus content of the lipid free fraction. It has been demonstrated by Knight (7) that both types of nucleic acid are present since a nucleic acid fraction separated from over 2 gm. of purified virus gave positive color reactions for both pentose and desoxypentose.

Knight concluded from his analyses that the virus contained about 2.3% pentose nucleic acid, later revised to 3.0% (8), based on the use of the orcinol color test for pentose. While no figures were given, Knight mentioned that there appeared to be 9 to 10 times more pentose than desoxypentose present. The initial work on the virus by Taylor (15) indicated the presence of 2.1% desoxypentose nucleic acid. Thus it would appear that there is not yet complete agreement on the analysis of the nucleic acid portion of the virus.

In order to separate the two nucleic acids of the radioactive virus to determine their isotope contents and also to estimate the two nucleic acids by an independent method, the technique of Schmidt and Thannhauser has been applied to the lipid free fraction of the virus. From the analyses it appeared that the virus contained about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. There was little or no phosphoprotein present. The same result was obtained with four different preparations of purified virus indicating, at least, that the technique of separation gave consistent results. Whether or not there was a clean separation of the two nucleic acids in the virus by this method cannot be deduced from phosphorus analyses alone. It has been demonstrated in the course of the present work that yeast and calf thymus nucleic acids can be separated almost quantitatively by the Schmidt and Thannhauser method, and similar results have been obtained with sheep liver pentose nucleic and calf thymus nucleic acids by McCarter and Steljes (9). However, it is not certain that the nucleic acids of influenza virus behave in the same manner as other nucleic acids when submitted to the action of dilute alkali.

In carrying out color tests on the separated nucleic acids of the virus in an attempt to confirm the above findings, it was observed that both the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose might be subject to interference from other constituents of the virus. While the results of the colorimetric tests do not, therefore, give unqualified support for the estimations of the nucleic acids from phosphorus analyses of the separated acids, they are in agreement with the phosphorus analyses to the extent that they indicate the presence of much more pentose than desoxypentose nucleic acid.

The observation that in influenza virus containing P^{32} both phospholipid and nucleic acid fractions were labelled furnishes evidence, in addition to that already presented (5), that the isotope was incorporated into the structure of the virus. The nucleic acid fraction had a specific activity about four times that of the phospholipid, practically all the P^{32} of the nucleic acids appearing in the pentose nucleic acid. At the beginning of the work it was hoped that labelled influenza virus might be of assistance in determining whether the nucleic acids of the virus were synthesized after infection of the cell or incorporated into the virus directly from the constituents of the cell. However, the low specific activities of the radioactive virus preparations so far obtained, in addition to the relatively short half life of P^{32}, make analytical

work difficult. A method must be found for greatly increasing the specific activity of the virus before the problem will be amenable to this method of attack.

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THE EFFECTS OF HIGH FAT DIETS AND COLD ENVIRONMENT ON THE ASCORBIC ACID CONTENT OF THE BROWN ADIPOSE TISSUE[1]

By Edouard Pagé and Louis-Marie Babineau

Abstract

At room temperature, the ascorbic acid content of the brown adipose tissue is doubled when rats are fed a high fat diet. It is nevertheless lower than in tissues studied by others under somewhat similar conditions. In cold adapted rats, the brown body is considerably hypertrophied and the ascorbic acid content is from four to eight times higher than at room temperature. Under our experimental conditions, rats exposed to cold doubled the weight of their perirenal fat with little or no change in total body weight. It is concluded that cold stimulates fat metabolism and that both ascorbic acid and the brown adipose tissue are involved in the process.

Introduction

We have already shown that prolonged exposure of rats to cold causes a considerable increase in the weight of the interscapular brown fat. This hypertrophy was associated with a rise in water and in nonfat dry matter content, and it was suggested that brown adipose tissue has some physiological function different from that of ordinary depot fat and that its activity is greatly enhanced in cold adapted animals (7). According to Wells (12), "Hoepke and Nikolaus (6) have recently demonstrated the probable presence of vitamin C in the hibernating gland of the hedgehog". Dugal and Thérien (3) have established the fact that the ascorbic acid concentration of several tissues increases in animals adapted to cold and it seemed opportune in the course of further experiments to measure the concentration of this vitamin in brown adipose tissue.

Experimental

Four groups of 30 male albino rats each were used. Two groups received a low fat and a high fat ration, respectively, at room temperature while the two other groups received the same rations in the cold. The composition of the low fat ration was as follows: casein (untreated), 15.0; sucrose, 74.0; mineral salts, 4.0; Celluflour, 2.0; Mazola oil, 2.5; wheat germ oil, 2.5 gm. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.5; pyridoxine hydrochloride, 0.5; nicotinic acid, 3.0; calcium pantothenate, 3.0; inositol, 10.0; 2-methyl-1, 4-naphthoquinone, 0.1; and choline chloride, 150 mgm. The high fat ration contained 40% fat as follows: Crisco, 25; Mazola oil, 10; and wheat germ oil, 5 gm. The level of other constituents was so adjusted at the expense of sucrose as to be similar to that of the low fat ration *on a calory basis*. A vitamin A and D supplement was given weekly by mouth.

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Average initial weight was 282 gm. for all groups. Two groups were put in the cold room at a temperature of about 8° C. (±2° C.) for the first 16 days. They were then exposed to temperatures varying between 0° and +3° C. until the end of the experiment when they were killed by decapitation between the 117th and 127th day. Only six rats could be sacrificed each day because of the many tissues taken for analysis. However, an equal number was taken from each group every day. Rats kept at room temperature were similarly sacrificed, some before and the others immediately after the cold adapted animals. Twenty additional rats were sacrificed at the beginning of the experiment and the fresh weights of various tissues were determined. Ascorbic acid was determined in the brown fat from some of the rats at room temperature and from others in the cold. Others were preserved for different analyses. The ascorbic acid was determined according to the method of Bessey and King (1). The perirenal fat (left side) was dissected and weighed in order to estimate the degree of fatness of the animals.

Results

Changes in body weight are shown in Table I. These figures do not take into account the animals which died in the cold room and a few, at room temperature, which were discarded at an early date because of apparent ill

TABLE I

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON BODY WEIGHT CHANGES IN THE RAT

(Average initial body weight: 282 gm. for all groups)

	Number of rats	Final body weight (gm.)	Changes in body weight (gm.)	Significance of differences between groups (Value of "t")
A—Room temperature				
Low fat group	25	378	+ 96 ± 4.95*	3.11
High fat group	26	411	+129 ± 9.38	
B—Cold room				
Low fat group	24	277	− 5 ± 5.05	2.66
High fat group	24	295	+ 13 ± 4.16	

* *Standard error:* $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

health. It should also be made clear that although the animals in the cold room made little or no overall gain, they were actually recovering at the end some of the weight lost initially. They can therefore be considered as acclimatized.

Larger gains in body weight were made by the rats kept on a high fat diet. This confirms for rats exposed to cold the previous findings of Dugal, Leblond, and Thérien (2). At room temperature, where the difference is marked, the excess gain in body weight appears to be largely due to increased fat storage, if the weight of the perirenal fat is any indication of total fat depots (Table II).

TABLE II

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON PERIRENAL FAT (LEFT SIDE)

	Number of rats	Perirenal fat (gm.)	Value of "t"**	Perirenal fat per 100 gm. B.W.	Value of "t"
A—Room temperature					
Initial control group*	20	0.78 ± 0.10		0.27 ± 0.03	
Low fat group	25	4.57 ± 0.34	4.96	1.19 ± 0.07	4.98
High fat group	26	7.49 ± 0.48		1.79 ± 0.09	
B—Cold room					
Low fat group	24	1.58 ± 0.14		0.57 ± 0.05	
High fat group	24	1.89 ± 0.14	1.57	0.64 ± 0.05	1.00

* *This group was representative of the others and was sacrificed on the day the experiment was initiated.*

** *Values of "t" exceeding 6.60 are found in all cases when comparing the low or high fat group in the cold with its control group at room temperature.*

TABLE III

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON THE BROWN ADIPOSE TISSUE

	Number of rats	Brown fat (gm.)	Value of "t"*	Brown fat per 100 gm. B.W.	Value of "t"
A—Room temperature					
Initial control group	20	0.26 ± 0.015		0.09 ± 0.026	
Low fat group	25	0.80 ± 0.03	1.64	0.21 ± 0.01	0.06
High fat group	26	0.91 ± 0.06		0.22 ± 0.01	
B—Cold room					
Low fat group	24	1.30 ± 0.05	1.96	0.47 ± 0.02	0.90
High fat group	24	1.52 ± 0.10		0.51 ± 0.03	

* *Values of "t" exceeding 5.20 are found in all cases when comparing the low or high fat group in the cold with its control group at room temperature.*

This effect of high fat rations has been demonstrated by many authors, notably Reed, Yamaguchi, Anderson, and Mendel (10), Pickens *et al.* (8), Williams *et al.* (13), and we had ourselves noted it previously (7). In the cold, the amount of perirenal fat is much less than at room temperature, nor is the difference between diets of any statistical significance. Surprisingly enough, the perirenal fat (left side) amounts to 0.6% of the total body weight in both groups exposed to cold as compared to 0.3% in the control rats killed initially (Table II), although average body weights are nearly the same in all cases. While it has been known for a long time that the water content of the body decreases in the cold, it is interesting to find in the present instance what appears to be a corresponding replacement of water loss by gain in fat.

It is seen in Table III that the weight of the interscapular brown adipose tissue increases with age and body weight at room temperature, with no significant differences between groups. In the cold, the increase is so marked that the brown fat weighs over 60% more, in absolute values, than in the larger control rats kept at room temperature and over twice as much on a body weight basis, irrespective of the ration.

Table IV shows the ascorbic acid content of brown adipose tissue in seven rats from each group at room temperature and in six rats from each group in

TABLE IV

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON THE ASCORBIC ACID CONTENT OF THE BROWN ADIPOSE TISSUE

	Room temperature	Cold room	Increase in the cold (%)	Value of "*t*"
A—Ascorbic acid concentration (γ/gm.)				
Low fat group	65.3 ± 14.8*	305.6 ± 41.1	368	5.50
High fat group	119.4 ± 19.5	353.7 ± 36.9	196	5.62
Increase on high fat ration (%)	83	16		
Value of "*t*" (low vs. high fat)	2.22	0.89		
B—Ascorbic acid content (γ)				
Low fat group	52.4 ± 6.4	437.5 ± 64.1	735	5.98
High fat group	103.2 ± 11.4	455.2 ± 62.7	341	5.52
Increase on high fat diet (%)	97	4		
Value of "*t*" (low vs. high fat)	3.88	0.19		

* *Standard error:* $\sqrt{\frac{\Sigma d^2}{n(n-2)}}$

the cold. These rats were taken at random and it so happened that in the cold, the average weight on the low fat diet was higher than the average weight for the whole groups (1.43 gm. as against 1.30), while it was lower for the high fat group (1.28 gm. as against 1.52 for all animals). It is nevertheless abundantly clear that prolonged exposure to cold increases considerably the ascorbic acid content of the brown adipose tissue. It is further noted that at room temperature, the ascorbic acid content is approximately doubled on the high fat ration.

Discussion

Variations in the weight of the perirenal fat with diet and temperature will be discussed in a separate paper dealing with the quantitative relationship between this tissue and other fat deposits.

It is sufficient to note here: (*a*) that the weight of the perirenal fat is much less in the cold than at room temperature although in the former case, the brown adipose tissue is considerably hypertrophied; (*b*) that following exposure to cold the animals increased their fat stores without gaining weight. Thus, initial loss in body weight in the cold may represent to a large measure a loss of water which is gradually compensated by increased fat deposition.

From the increase in weight of the brown adipose tissue in the cold and from the much higher concentration in ascorbic acid, it can safely be concluded that cold stimulates the activity of this tissue. If we compare our values for ascorbic acid with those reported by Dugal and Thérien (3) for other rat tissues under somewhat similar conditions, we find that at room temperature the concentration in brown fat is much less than in the testes, kidneys, or liver. In the cold, it becomes equal to that of the liver and is exceeded only by that of the adrenals. It seems, therefore, that insofar as functions connected with ascorbic acid are concerned, *the brown adipose tissue is relatively unimportant at room temperature but displays in the cold an activity level comparable to that of the liver.*

Dugal and Thérien (3) have already shown the close relationship existing between adaptation to cold, adrenal function, and ascorbic acid. On the other hand, Fawcett and Jones (5) have demonstrated by cytological studies that the "maintenance of the normal complement of lipid in brown adipose tissue depends upon the functional integrity of the adrenal cortex". It is, therefore, more than probable that the *stimulating effect of cold on this tissue is mediated through the adrenals.*

There is also good reason to believe that the brown adipose is involved in fat metabolism. In hibernating animals brown fat attains its maximum size just prior to hibernation and at a time when vast stores of fat are being accumulated. It degenerates during winter sleeps (9). This "hibernating gland" may therefore be concerned with *preparation* for, rather than *maintenance* of hibernation. Fawcett and his associates (4, 11) have brought forward excellent evidence that it may be a preferred site of fat synthesis: thus, in hyperglycemic but nondiabetic rats, as well as in normal or diabetic

animals after the administration of insulin, the deposition of glycogen prior to its conversion into fat is far greater in brown adipose tissue than in ordinary depot fat.

Finally, Dugal and Thérien (3) have shown that at room temperature, a high fat ration always leads to a higher concentration of ascorbic acid in tissues, as compared to a low fat ration, this difference tending to disappear in the cold where concentrations are much higher. Our own results fully confirm this finding with the additional information that increase in ascorbic acid concentration in the cold is greater in the brown adipose tissue than in any other organs studied. Because of the obvious relationship between this tissue and fat metabolism, we feel justified in considering that the particularly marked rise in its ascorbic acid content in the cold indicates that *this vitamin is linked with some phase of fat metabolism.*

Summary and Conclusion

In rats adapted to a cold environment, there occurs a considerable hypertrophy of the brown adipose tissue and an even higher rise in its ascorbic acid content. Under our experimental conditions, these rats increased their fat stores with little or no change in body weight. At room temperature, the ascorbic acid content of the brown adipose tissue is doubled on a high fat ration. It is suggested, on the basis of these findings and from other considerations discussed above, that this tissue is involved in fat metabolism, that exposure to cold stimulates its activity and finally, that ascorbic acid is intimately linked with some phase of fat metabolism.

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the cold. These rats were taken at random and it so happened that in the cold, the average weight on the low fat diet was higher than the average weight for the whole groups (1.43 gm. as against 1.30), while it was lower for the high fat group (1.28 gm. as against 1.52 for all animals). It is nevertheless abundantly clear that prolonged exposure to cold increases considerably the ascorbic acid content of the brown adipose tissue. It is further noted that at room temperature, the ascorbic acid content is approximately doubled on the high fat ration.

Discussion

Variations in the weight of the perirenal fat with diet and temperature will be discussed in a separate paper dealing with the quantitative relationship between this tissue and other fat deposits.

It is sufficient to note here: (*a*) that the weight of the perirenal fat is much less in the cold than at room temperature although in the former case, the brown adipose tissue is considerably hypertrophied; (*b*) that following exposure to cold the animals increased their fat stores without gaining weight. Thus, initial loss in body weight in the cold may represent to a large measure a loss of water which is gradually compensated by increased fat deposition.

From the increase in weight of the brown adipose tissue in the cold and from the much higher concentration in ascorbic acid, it can safely be concluded that cold stimulates the activity of this tissue. If we compare our values for ascorbic acid with those reported by Dugal and Thérien (3) for other rat tissues under somewhat similar conditions, we find that at room temperature the concentration in brown fat is much less than in the testes, kidneys, or liver. In the cold, it becomes equal to that of the liver and is exceeded only by that of the adrenals. It seems, therefore, that insofar as functions connected with ascorbic acid are concerned, *the brown adipose tissue is relatively unimportant at room temperature but displays in the cold an activity level comparable to that of the liver.*

Dugal and Thérien (3) have already shown the close relationship existing between adaptation to cold, adrenal function, and ascorbic acid. On the other hand, Fawcett and Jones (5) have demonstrated by cytological studies that the "maintenance of the normal complement of lipid in brown adipose tissue depends upon the functional integrity of the adrenal cortex". It is, therefore, more than probable that the *stimulating effect of cold on this tissue is mediated through the adrenals.*

There is also good reason to believe that the brown adipose is involved in fat metabolism. In hibernating animals brown fat attains its maximum size just prior to hibernation and at a time when vast stores of fat are being accumulated. It degenerates during winter sleeps (9). This "hibernating gland" may therefore be concerned with *preparation* for, rather than *maintenance* of hibernation. Fawcett and his associates (4, 11) have brought forward excellent evidence that it may be a preferred site of fat synthesis: thus, in hyperglycemic but nondiabetic rats, as well as in normal or diabetic

animals after the administration of insulin, the deposition of glycogen prior to its conversion into fat is far greater in brown adipose tissue than in ordinary depot fat.

Finally, Dugal and Thérien (3) have shown that at room temperature, a high fat ration always leads to a higher concentration of ascorbic acid in tissues, as compared to a low fat ration, this difference tending to disappear in the cold where concentrations are much higher. Our own results fully confirm this finding with the additional information that increase in ascorbic acid concentration in the cold is greater in the brown adipose tissue than in any other organs studied. Because of the obvious relationship between this tissue and fat metabolism, we feel justified in considering that the particularly marked rise in its ascorbic acid content in the cold indicates that *this vitamin is linked with some phase of fat metabolism.*

Summary and Conclusion

In rats adapted to a cold environment, there occurs a considerable hypertrophy of the brown adipose tissue and an even higher rise in its ascorbic acid content. Under our experimental conditions, these rats increased their fat stores with little or no change in body weight. At room temperature, the ascorbic acid content of the brown adipose tissue is doubled on a high fat ration. It is suggested, on the basis of these findings and from other considerations discussed above, that this tissue is involved in fat metabolism, that exposure to cold stimulates its activity and finally, that ascorbic acid is intimately linked with some phase of fat metabolism.

Acknowledgments

The authors wish to thank Dr. Thérien from the Department of Acclimatization of the Institute for supervising the ascorbic acid determinations.

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FURTHER WORK ON THE NUTRITION OF DUCKLINGS

A.—LIPOTROPIC FACTORS. B.—SULPHUR AMINO ACID REQUIREMENTS[1]

By J. M. Demers and R. Bernard

Abstract

Using a purified diet deficient in choline and containing 18% casein, it has been found that in the duckling: (*a*) betaine and aminoethanol have little or no lipotropic activity, are poor growth factors, and cannot prevent perosis; (*b*) monomethylaminoethanol and dimethylaminoethanol, on the other hand, have well marked lipotropic activity. Both substances stimulate growth and are antiperotic, dimethylaminoethanol being the most active of the two in these respects; (*c*) ducklings, like chicks, appear unable to methylate aminoethanol to any extent; (*d*) a level of 28% casein is more satisfactory than one of 18% in the prevention of fatty infiltration of the liver in ducklings.

Betaine and methionine have a definite lipotropic activity, when fed to ducklings along with a semipurified diet containing peanut meal as the only natural ingredient. The substitution of yellow corn meal to sucrose in the above semipurified diet inhibits the lipotropic activity of methionine, without affecting that of betaine.

The availability of a protein low in sulphur amino acids has resulted in the preparation of a more hypolipotropic diet. On this diet, ducklings presented livers containing, on the average, 24% of lipids. Methionine added to this diet is strongly lipotropic, but is without effect on growth. Methionine will promote growth only in presence of choline.

Ducklings like chicks and poults appear to be able to convert methionine to cystine. In presence of 0.4% cystine, approximately 0.5% methionine is required for normal growth.

Introduction

Using a purified diet containing 18% casein, Bernard and Demers (2) have shown that choline has a definite lipotropic action in ducklings. Under these conditions, betaine presented a slight lipotropic activity, but the results were not significant. On the other hand, methionine, when added to a purified diet containing 9% casein or to semipurified diet containing 15% casein and yellow corn meal, increased the total lipids and the severity of fatty infiltration of the livers. The purpose of this paper is to present the results of additional studies on lipotropism in ducklings and preliminary information on the sulphur amino acids requirements of this avian species.

Materials and Methods

The experimental procedures followed are similar to those already described in detail (2). These procedures deal with the preparation of the diets, care of the animals, the determination of liver lipids, and the preparation of microscopic sections of the liver.

An arbitrary fatty infiltration index (F.I. index) has been resorted to: microscopic examination of frozen sections of liver sections stained for fat

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permits the grading of fatty changes according to scale proposed by Drill and McCormick (4). An average score is calculated for each group and is designated as fatty infiltration index. This index varies from 0.0 to 4.0 and represents a mean fatty infiltration for each group of animals.

The composition of the basal diets used is given in Table I. Diets R-49, R-66, and R-73 resemble each other in being deficient in choline, though to a different degree. Diet R-73 is particularly hypolipotropic. Besides being

TABLE I

COMPOSITION OF BASAL DIETS

Ingredients	Basal diet number			
	R-49	R-66	R-73	R-76
	Composition			
	%			
Main ingredients				
Sucrose	47	50	51	51
Casein, Labco	18	10		
Isolated soybean protein*			28	28
Peanut meal**		15		
Liver fraction L***	4	4		
Fish oil blend†	2	2	2	2
Salts IV††	5	5	5	5
$CaH_4(PO_4)_2 2H_2O$	1	1	1	1
Ruffex	3	3	3	3
	Mgm. per 100 gm. of diet			
Vitamins				
Thiamine hydrochloride	0.4	0.4	0.4	0.4
Riboflavin	0.8	0.8	0.8	0.8
Calcium pantothenate	2.5	2.5	2.5	2.5
Nicotinic acid	4.0	4.0	4.0	4.0
Pyridoxine hydrochloride	0.4	0.4	0.4	0.4
p-Aminobenzoic acid	2.0	2.0	2.0	2.0
Biotin	0.02	0.02	0.02	0.02
Inositol	100.0	100.0	100.0	100.0
2-Methyl naphthoquinone	0.1	0.1	0.1	0.1
α-Tocopherol	10.0	10.0	10.0	10.0
Pteroylglutamic acid			0.1	0.1
Choline chloride				300.0

*"*Alpha protein*". *The Glidden Company, Chicago, Ill.*

** *Expeller process, 45% protein. Planters Edible Oil Co., Suffolk, Va.*

*** *A source of the unknown vitamins of the B-complex. Wilson Laboratories, Chicago, Ill.*

† *Vadol Type No. IV, 3000 A, 400 D (A.O.A.C. chick units). Blended fish oils fortified with vitamin D_3. Ayerst, McKenna and Harrison, Montreal, Que.*

†† *Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B. J. Biol. Chem. 138 : 459. 1941.*

choline-free, it contains a protein which is low in cystine and methionine. The purified diet R-49 contains about 0.004% choline (calculated), i.e., that brought by the Liver Fraction L. The simplified (semipurified) diet R-66 has a choline content (calculated) about 10 times that of the preceding diet. This is accounted for by the peanut meal and the Liver Fraction L. Finally, diet R-76 includes all the ingredients of diet R-73 plus 0.3% added choline chloride and is well suited for the study of cystine and methionine requirements.

Results and Discussion

A. Lipotropic Factors

(*a*) The results of the first three series of experiments are presented in Fig. 1 and Table II. Each experiment includes groups of animals started on the

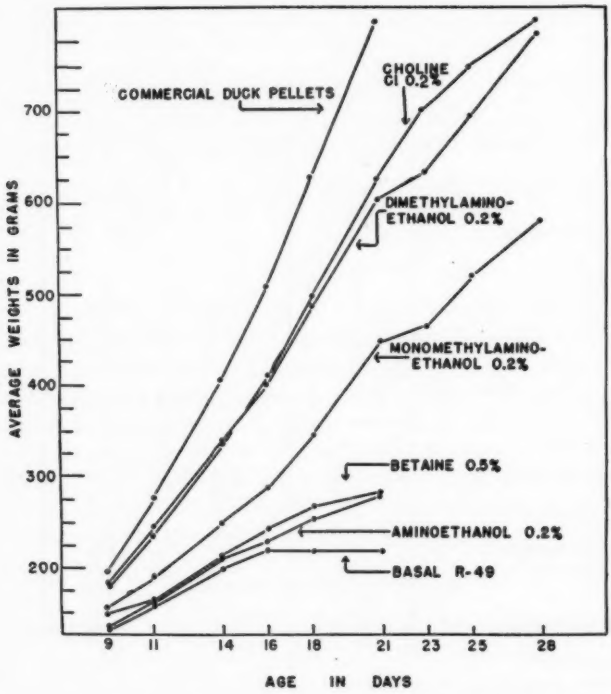

FIG. 1. *Growth curves of ducklings fed different lipotropic factors.*

same day. Using purified diet R-49, the lipotropic effect of betaine, aminoethanol, monomethylaminoethanol, and dimethylaminoethanol were studied, as well as the effect of raising the casein level. Observations were also made on growth and on incidence of perosis.

TABLE II

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO AN 18% CASEIN, PURIFIED DIET

Expt.*	Supplement to basal diet R-49	F.I. index**	Liver lipids, %***	t†	Birds with perosis / Birds on experiment	Average weight, gm. 5 days	Average weight, gm. 21 days
I	None	3.2	14.5 ± 1.91††		10/10	102	222 ± 12
	Betaine 0.5%	2.5	12.6 ± 2.40	0.40	9/10	105	284 ± 18
	Commercial duck pellets only	0.0	6.8 ± 0.13		0/6	83	800 ± 24
II	Aminoethanol 0.2%	3.1	12.5 ± 1.85	0.73	6/8	104	280 ± 16
	Choline chloride 0.3%	1.4	7.4 ± 0.76	3.48	0/8	86	574 ± 21
	Choline chloride 0.3% Casein, Labco, 10%	0.6	6.7 ± 1.34	3.33	0/8	85	601 ± 30
III	Choline chloride 0.2%	1.4	8.2 ± 0.43		0/8	89	625 ± 22
	Monomethylaminoethanol 0.2%	2.1	8.2 ± 0.97		5/8	95	447 ± 24
	Dimethylaminoethanol 0.2%	1.6	8.5 ± 0.33		2/8	95	604 ± 35

* *Birds of Experiment III were killed at 28 days, others at 21 days.*

** *Fatty infiltration index of the liver.*

*** *Per cent wet weight.*

† $t > 2.30$ *significant by comparison with control group.*

†† *Standard error* $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

1. Betaine. Previous work (2) on ducklings indicated that betaine has a slight lipotropic activity though the results were not statistically significant. The investigation was repeated, and it was found that this quaternary amine has little or no lipotropic activity and has no antiperotic properties. A small but significant stimulation of growth was noticed.

2. Aminoethanol, monomethylaminoethanol, and dimethylaminoethanol.

In Table II, the percentages of these amines refer to the free bases. However, they were neutralized with concentrated hydrochloric acid prior to their addition to the basal diet.

It is well known that there exist striking differences between the rat and the chick in respect of the utilization of these precursors of choline.

In 1941, Stetten (14) found in the rat, that aminoethanol can accept labile methyl groups for the synthesis of choline. In the chick, on the contrary, Jukes (5) observed that this primary amine cannot serve as a precursor of choline, when added to a purified diet enriched in methionine.

Using a semipurified diet containing yellow corn meal and peanut meal as natural ingredients, McGinnis, Norris, and Heuser (11) noticed in chicks that aminoethanol does not improve growth and has very slight antiperotic activity. On the other hand, Kummerow, Weaver, and Hornstead (10) recently reported that the same amine is antiperotic and stimulates growth, when fed along with a natural diet rich in fats.

Jukes and coworkers (6, 7) have demonstrated with chicks that dimethylaminoethanol is as efficient as choline in the prevention of perosis, but slightly inferior to it as a growth factor. Monomethylaminoethanol is antiperotic, but promotes growth only in the presence of methionine. These last authors conclude that the chick is unable to methylate aminoethanol, but can accomplish the second step in the synthesis of choline, that is, the transformation of monomethylaminoethanol into dimethylaminoethanol.

From the standpoint of comparative physiology, it was thus of interest to investigate the effect of feeding these amines to ducklings, particularly with respect to their influence on liver lipids.

The present studies with ducklings indicate that aminoethanol (Table II, Expt. II) has little or no lipotropic activity, that it is a poor growth factor and that it does not prevent perosis. It appears that ducklings are similar to chicks in being unable to methylate aminoethanol. Monomethylaminoethanol and dimethylaminoethanol (Expt. III) exhibit a lipotropic activity equal to that of choline. Dimethylaminoethanol is as good as choline in promoting growth, and only slightly inferior to the latter in antiperotic properties. On the other hand, 60% of the birds treated with monomethylaminoethanol showed symptoms of perosis and the average growth for the group was only two-thirds that of the control group fed choline chloride.

Working with chicks, Jukes *et al.* (7) found that monomethylaminoethanol prevents perosis without any improvement on growth. It appears that chicks and ducklings react differently when fed a diet supplemented with monomethylaminoethanol.

3. Effect of increasing the casein content of the diet on fatty infiltration of the liver.

In a preceding study (2), the authors have reported that the supplementation with 0.3% choline chloride of diet R-49 containing 18% casein did not bring the percentage of total lipids entirely back to normal, and that fatty infiltration was still persistent. Consequently, an attempt was made to assess the lipotropic value of casein by increasing the percentage of this protein by 10%. It will be seen (Expt. II) that the group of ducklings fed the 28% casein diet presented an average of 6.7% for liver lipids. This is as low as that of the group fed the commercial diet. Moreover, the fatty infiltration of the liver nearly disappeared. Growth was good and nearly equal in both groups.

It is thus concluded, that the purified diet containing 18% casein and supplemented with 0.3% choline chloride is still deficient in one or more lipotropic factors.

(*b*) Lipotropic activity of betaine and methionine in presence of semipurified diets.

The following experiments were suggested by the observations of McGinnis, Norris, and Heuser (12) in chicks. These authors reported that betaine or methionine do not improve in any way the nutritive value of a purified diet. On the other hand, when the same substances were added to a semipurified diet containing peanut meal or yellow corn meal, they improved growth markedly and were effective in preventing perosis.

A similar study was undertaken in ducklings making use of semipurified diet R-66 which contained 15% peanut meal as the natural ingredient. The protein content of this basal diet is approximately 16%.

Table III shows that the addition of 0.5% betaine (Expt. IV) reduces the liver lipids to 9.2% as compared with 16.8% for the unsupplemented group.

TABLE III

EFFECT OF PEANUT MEAL AND YELLOW CORNMEAL ON THE LIPOTROPIC ACTIVITY OF BETAINE AND METHIONINE

Expt.	Group No.	Supplement to basal diet R-66	F.I. index	Liver lipids, %	t	Birds with perosis / Birds on experiment	Average weights, gm.	
							5 days	28 days
IV	1	None	3.5	16.8 ± 1.06		8/8	105	770 ± 39
	2	Betaine 0.5%	1.9	9.2 ± 0.54	6.39	6/8	95	679 ± 42
	3	DL-methionine 0.5%	1.5	8.7 ± 0.49	6.90	6/8	96	801 ± 48
V	4	Corn meal 50%	0.9	8.4 ± 0.93		5/8	96	986 ± 46
	5	Corn meal 50% Betaine 0.5%	0.3	7.0 ± 0.32	1.42	6/8	95	820 ± 29
	6	Corn meal 50% DL-methionine 0.5%	2.0	12.3 ± 1.75	2.88	8/8	85	816 ± 41

Comparison of liver lipids: Groups 1 and 4, $t = 5.94$.
" 2 and 5, $t = 3.45$.
" 3 and 6, $t = 3.33$.

Methionine is as effective as betaine, if not more, in this respect. However, both groups of birds still showed evidence of slight fatty infiltration. These results constitute the first evidence of the lipotropic activity of betaine and methionine in ducklings.

Substitution of yellow corn meal to the sucrose of basal diet R-66 (Expt. V) lowered the liver lipids from 16 to 8%. This lipotropic effect of the yellow corn meal is probably due to its choline content. It was surprising to find that the simultaneous addition of yellow corn meal and methionine favors the production of fatty livers. This new information confirms the antilipotropic effect of methionine in presence of yellow corn meal as noticed in a previous work (2). It is suggested that the zein content of the corn meal may cause an imbalance in amino acid makeup of the diet which is reflected by the appearance of fatty livers. Zein is known to contain large quantities of leucine and

glutamic acid. Furthermore, Beveridge *et al.* (3) have showed that the lipotropic effect of a diet is determined not only by its sulphur-containing amino acids but also by its adequacy in other respects, particularly the sulphur-free amino acids in the protein.

Since the majority of the birds in Experiments IV and V were perotics, it is suggested that if any choline was synthesized from betaine or methionine, it must have been used up for some other purpose, probably the lipotropic function. Finally, Table III shows that all groups grew well, especially the fourth one which presented the highest average weight at 28 days of age.

In summary, the experiments with semipurified diets have revealed that betaine and methionine are lipotropic in presence of peanut meal. One may surmise that this natural ingredient contains a suitable methyl acceptor or that it corrects an amino acid imbalance. The replacement of sucrose by yellow corn meal in diet R-66 did not alter the lipotropic activity of betaine but inhibits that of methionine and even renders it antilipotropic.

(*c*) Study of the lipotropic activity of cystine and methionine using a diet containing isolated soybean protein.

In a last series of experiments on fatty infiltration of the liver of ducklings a more hypolipotropic diet (R-73) was made use of. The Liver Fraction L of previous diets was dispensed with and the casein replaced with a soybean protein known to be deficient in cystine and methionine (9). Table IV shows an average of 24% for the liver lipids of the unsupplemented group. This

TABLE IV

EFFECT OF CYSTINE AND METHIONINE WHEN ADDED TO A CHOLINE-FREE DIET, CONTAINING 28% ISOLATED SOYBEAN PROTEIN

Supplement to basal diet R-73	F.I. index	Liver lipids, %	*t*	Birds with perosis / Birds on experiment	Average weights, gm.	
					5 days	21 days
None	3.6	24.2 ± 3.98		5/8	107	193 ± 6
L-cystine 0.5%	2.9	15.3 ± 2.75	1.84	8/8	105	162 ± 5
DL-methionine 0.5%	0.8	6.6 ± 0.42	4.39	8/8	88	209 ± 9

value exceeds that of the similar groups of preceding experiments by 8 or 11% respectively and confirms the marked hypolipotropic nature of basal diet R-73. It should be recalled, however, that a 9% casein diet (2) produced livers containing on the average, 34% lipids for the basal group.

The group of ducklings fed the cystine supplement had a liver lipid value of 15%, though the results are not significant when compared to the control group. This result is surprising, for cystine is recognized in the rat (8, 13) as antilipotropic, especially when added to diets low in protein and rich in fats.

Methionine at the level of 0.5% was very effective as a lipotropic factor. The liver lipids of this group being 6.6% with a low fatty infiltration index of 0.8%. This last experiment further shows that the lipotropic effect is related to other constituents of the diet particularly the quality and quantity of the protein used.

Table IV further reveals that on a choline-free diet, methionine does not improve growth, while cystine has a definite retarding effect. Finally, most ducklings of the three groups were perotic.

This last series of experiments with basal diet R-73 brings additional evidence for the lipotropic activity of methionine in ducklings. It is surprising to note that the action of methionine is limited to the prevention of fatty livers to the exclusion of growth. If the lipotropic effect of this amino acid is exerted through the biosynthesis of choline, it is possible that this latter substance is used up entirely for the removal of fat from the livers and that none is left for growth and prevention of perosis. It would be of interest to know which of these functions has priority over the others.

B. The Sulphur Amino Acid Requirements of Ducklings

Using a basal diet (R-76) containing 28% isolated soybean protein and a sufficient amount of choline, it was possible to investigate the requirements of ducklings for cystine and methionine. The basal diet resembles that used by Kratzer *et al.* (9) in their study with turkey poults. According to these authors, it contains 24% crude protein, 0.36% methionine, and 0.05% cystine, and growth is limited by methionine and cystine deficiencies.

The results are presented in Table V and Fig. 2. The basal diet alone or supplemented with 0.5% cystine are inadequate for growth. Excellent growth

TABLE V

SULPHUR AMINO ACID REQUIREMENTS OF DUCKLINGS

Supplement to basal diet R-76	F.I. index	Liver lipids, %	Birds with perosis / Birds on experiment	Average weights, gm.		*t*
				4 days	22 days	
None	1.0	7.1 ± 0.32	2/5	103	253 ± 11	
DL-methionine 0.1%	1.1	7.4 ± 0.32	1/7	102	464 ± 25	7.38
DL-methionine 0.3%	0.7	6.9 ± 0.14	0/6	94	541 ± 39	7.08
DL-methionine 0.5%	0.1	7.0 ± 0.05	3/7	85	402 ± 31	4.45
L-cystine 0.5%	0.1	6.4 ± 0.80	0/8	94	296 ± 25	1.64
L-cystine 0.5% DL-methionine 0.1%	0.3	7.0 ± 0.39	1/7	82	536 ± 30	8.73

was obtained with the basal diet enriched with 0.1 or 0.3% methionine, the last level being more satisfactory. It thus appears that like chicks, turkeys, and other animals, ducklings are able to convert methionine into cystine and

that cystine may not substitute for methionine. It should be emphasized, however, that methionine improves growth only in the presence of an adequate supply of choline. It is surprising to note that a level of 0.3% methionine is

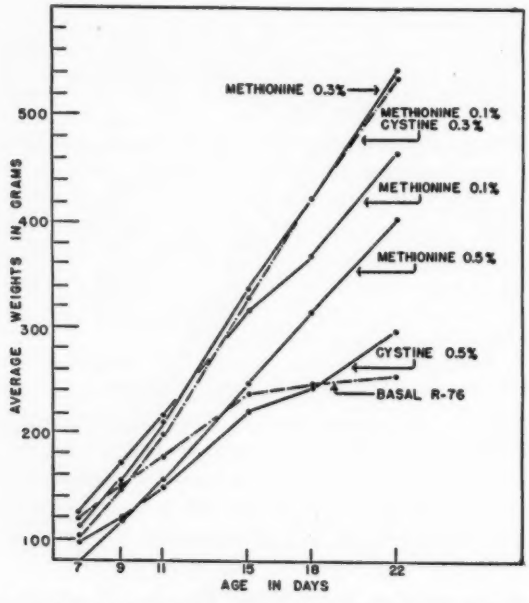

FIG. 2. *Effect of different levels of methionine and cystine on growth of ducklings.*

more satisfactory for growth than that of 0.5%. An excess of methionine may create an amino acid imbalance which is translated by inhibition of growth and higher incidence of perosis as observed in the fourth group of birds. Finally, addition of 0.3% cystine and 0.1% methionine to the basal diet gives excellent growth equal to that obtained with 0.3% methionine only. Thus, the simultaneous addition of the two sulphur amino acids results in a significant economy in methionine.

Examination of the liver fatty infiltration indices suggest that methionine and cystine add to the lipotropic effect of choline alone except for the group which received 0.1% methionine. However, this is not corroborated by the liver lipid values which are close to normal in all groups.

Taking into account the sulphur amino acid contents of the basal diet, it is suggested that in the relative absence of cystine, the methionine requirement of ducklings are of the order of 0.8% cystine. In the presence of 0.4% cystine, a level of 0.5% methionine is apparently adequate for normal growth. These values are close to those found by Kratzer *et al.* (9) for turkey poults and Almquist (1) for chicks.

Acknowledgments

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AN ANTIBIOTIC PRODUCED BY *MICROCOCCUS EPIDERMIDIS*[1]

By L. J. Loeb, A. Moyer, and R. G. E. Murray

Abstract

A stable antibiotic was produced by a strain of *Micrococcus epidermidis* that showed a wide range of activity against Gram-positive organisms. A mucoid *Streptococcus pyogenes* was used as test organism. This strain could be made resistant by being grown in increasing concentrations of antibiotic but the organism reverted to its original susceptibility immediately on transfer to medium without antibiotic. There was no antiluminescent activity when tested on *Photobacterium fischeri*. The test organism was not lysed by the antibiotic. The active substance was dialyzable, was remarkably heat stable, and was soluble only in water or, providing water was present, in solvents that were completely miscible with water. Purification was successful only to the extent of removing a number of inactive fractions by differential solubilities. The activity was destroyed by trypsin but not by pepsin. The physical and chemical data make it probable that the substance is a polypeptide of low molecular weight.

Two strains of *Micrococcus epidermidis* have been described (4) that produced a stable antibiotic. The activity was restricted to a range of Gram-positive organisms and was diffusable through cellophane. The results of further study of the antibiotic produced by one of these strains (2921) is presented in this paper.

Methods

The media used and the general methods of production and titration have been described by Murray and Loeb (4). The antibiotic was produced by a cellophane sac technique in Difco nutrient broth supplemented by equal amounts of Difco proteose peptones No. 2 and No. 3 added to 1%. By this method a cell-free broth was obtained. A mucoid *Streptococcus pyogenes* (M. Strep. 9) was used as the test organism in plate tests on blood agar and in the dilution assays in dextrose broth.

Observations

Production

Assay during production showed that the titer rose to a maximum and remained at that level. After 14 days' incubation at 37° C. the broth usually attained a titer greater than 1/200 and was harvested as "crude antibiotic". This material was stored in screw-top vessels ready for subsequent treatment. No drop in activity has been noted during storage. In fact, aliquots stored at room temperature and at 4° C. have maintained a constant titer for more than 18 months.

Proteose-peptone seemed to provide some factor necessary for satisfactory production of the antibiotic. Production in a nutrient broth base was compared

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ont. Aided by a grant from the National Research Council of Canada.

to the same medium with the addition of 0.5% and 1.0% of proteose-peptone (Fig. 1). These supplements allowed a more rapid production and an increased titer.

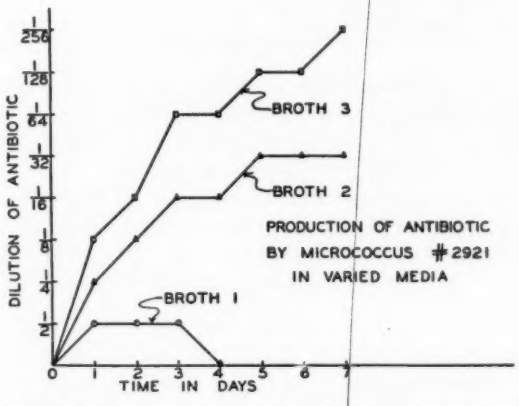

FIG. 1. *A graph showing titers of antibiotic produced by Micrococcus epidermidis (2921) in varied media.*

Broth 1: Nutrient broth.
Broth 2: Nutrient broth with 0.5% proteose-peptone.
Broth 3: Nutrient broth with 1.0% proteose-peptone.

BIOLOGICAL ACTIVITY

The antibiotic was active against a number of Gram-positive bacteria, as shown by the streak plate tests (4). *Corynebacterium diphtheriae* (five strains) and *Mycobacterium tuberculosis* (H37RA and B.C.G.) were not affected nor were any of the Gram-negative organisms that were tested. It was found also that a bactericidal action on the test *Streptococcus* was exhibited when concentrations of antibiotic more than twice the minimum inhibitory level was used. There was no evidence of lysis and the cells did not show any change in staining character using Gram's method, even when suspended in a bactericidal concentration of antibiotic for four days. The test organism became more resistant on repeated passage through media containing gradually increasing concentrations of the crude antibiotic, but after one subculture to media without antibiotic the organism was again fully sensitive. Antibiotic 2921 does not have any antiluminescent activity against *Photobacterium fischeri*, a property (3) that is peculiar to some antibacterial agents.

PHYSICAL AND CHEMICAL PROPERTIES

Stability

The stability of the crude antibiotic at room and refrigerator temperature has already been noted. It was unaffected by autoclaving for 20 min. at 121° C. or by boiling for 30 min. at pH 2, pH 7, and pH 12.

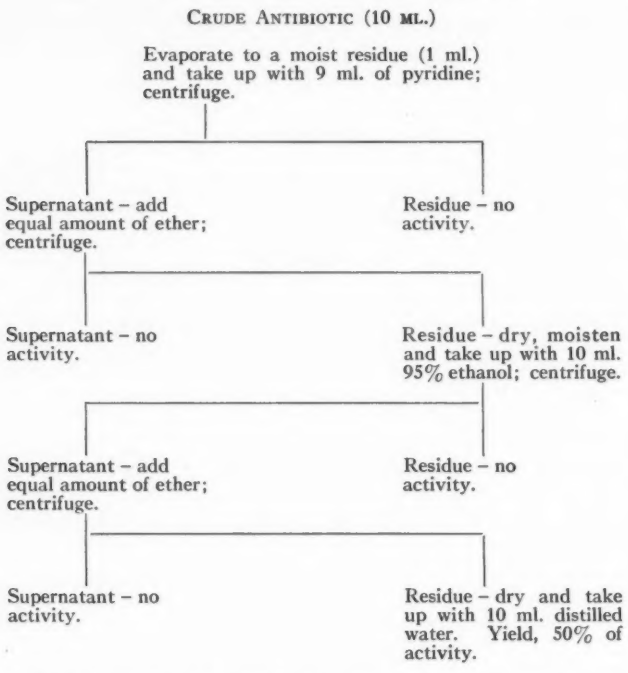

FIG. 2. *Flow sheet diagram of partial purification of the crude antibiotic from Micrococcus epidermidis.*

Solubility

The activity was readily soluble in water at all pH reactions but could not be extracted with any of the common organic solvents from the dried residue of the crude antibiotic. However, the inhibitory substance was soluble in those solvents which are completely miscible with water if the dried residue was moistened slightly with water beforehand. These solvents included methyl, ethyl, and propyl alcohols, acetone, pyridine, and dioxane. It was not soluble in solvents that are only partially miscible or not miscible with water: ethyl ether, chloroform, butyl alcohol, ethyl acetate, carbon tetrachloride, or cyclohexane. The fractions were tested by filter paper disc method against M. Strep. 9 on blood agar plates.

Extraction and Purification

Considerable difficulty was experienced in attempting purification because of the solubility characteristics of the substance. The antibiotic was dialyzable through cellophane into both normal saline and distilled water, but only about 20% of the activity diffused in 24 hr. Phosphotungstic acid caused a heavy precipitate that included the antibiotic activity, but this residue proved to be

difficult to handle. Attempts to purify the antibiotic by partition chromatography were unsuccessful because no suitable solvent was found. The activity was adsorbed on "Norit" (activated animal charcoal) and was eluted by pyridine at pH 7, but not by water, 50% ethyl acetate or water-saturated butanol at pH 2, pH 7, or pH 10. The yield of antibiotic was low (10% of the activity) after elution with pyridine.

Using a moistened dried residue of the crude antibiotic, it was found that pyridine and ethyl alcohol precipitated impurities leaving the inhibitory substance in solution. The addition of ether caused a yellowish precipitate containing the antibiotic which could be redissolved in water from this residue. A process of partial purification seemed most feasible, giving approximately 50% yield of the inhibitory substance. A flow sheet diagram of this process is presented in Fig. 2. A brownish viscous fluid was obtained when the partially purified material was concentrated to 1/20th of the original volume.

Effect of Proteolytic Enzymes and Serum

The crude antibiotic was digested with 0.1% trypsin (Difco) at pH 8 and 1% pepsin (Difco 1 : 10,000) at pH 2, and plain broth was treated similarly as controls. The antibiotic was inactivated after one hour of incubation with trypsin at 37° C., while the aliquot digested with pepsin retained its activity. Incubation with sheep serum (1 : 1) for 18 hr. reduced the activity of the mixture by 50%.

Discussion

Antagonistic micrococci have been described for over 60 years. The early reports gave little indication of the nature of the substances, and strains were described in terms of antibacterial spectrum and possible applications. Inhibitory strains of *Micrococcus pyogenes* have been most frequently reported although Dujardin-Beaumetz (2) described a nonpathogenic *Micrococcus* that inhibited most Gram-positive organisms and many Gram-negative strains. Recently Su (5) described a nonpathogenic *Micrococcus* producing a dialyzable antibiotic that was heat stable and soluble in alcohol and chloroform. The activity of the purified fractions was not affected by horse serum, trypsin, or pepsin, and the action was bacteriostatic, affecting many Gram-positive organisms. The antibiotic produced by *Micrococcus epidermidis* described here does not correspond in antibacterial spectrum, physical or chemical properties to those described by Dujardin-Beaumetz and by Su.

The solubility of the antibiotic places it in a group of substances described by Waksman (6) which are "soluble in water at different reactions, and insoluble in ether. These substances usually represent polypeptides, proteins, organic bases, or adsorption compounds on protein molecules. Most of them have not been isolated in pure state".

The differential action of pepsin and trypsin may provide further information as to its chemical constitution. Bergmann (1) explained that trypsin can act on peptide links formed from the carboxyl group of either arginine

or lysine, but requires that the second amino group of the dibasic amino acid unit be free. Pepsin does not act on this linkage. This evidence suggests that the active substance may be a low molecular weight polypeptide that is heat stable and dialyzable through cellophane.

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OXYGEN UPTAKE OF RAT MAMMARY TISSUE SLICES[1]

By Jules Tuba, Herbert E. Rawlinson, and Lorna Glen Shaw

Abstract

An *in vitro* study has been made of the oxygen uptake of mammary gland tissue of female rats in various experimental states. Because of the very high proportion of fat in mammary tissue the values of Q_{O_2} are determined on a fat-free as well as a water-free basis, thus providing a more accurate measure of the oxygen consumption of this tissue. The oxygen utilization by mammary gland of pregnant animals is increased approximately three times over the activity in the normal, or resting, gland. This increase is maintained during lactation and a return toward normal levels occurs during postlactational involution. The response to *p*-phenylenediamine indicated that during lactation the increased energy requirements decreased the reserves of the cytochrome system in mammary tissue. There is a well developed mammary gland in adult male rats; but the average fat content and response to *p*-phenylenediamine of the tissue are almost identical with values for adult female rats. The use of *p*-phenylenediamine as a histological stain for the cytochrome system in mammary tissue is described.

Introduction

There has been comparatively little investigation of the respiratory activity of rat mammary gland tissue *in vitro*, probably because of the difficulties encountered in preparing the tissue for such studies. Kleiber, Smith, and Levy (5) measured the oxygen consumption of rat mammary tissue during pregnancy and lactation. They found on a dry-weight basis that mammary tissue from lactating rats manifested greater values of Q_{O_2} than tissue obtained from the pregnant animals. Attempts to correlate oxygen uptake with nitrogen content of the glands were unsuccessful because of the variable milk protein content. Folley and French (4) carried out a survey of the respiratory metabolism of slices of rat mammary tissue during pregnancy, lactation, and postlactational involution. Their values for Q_{O_2}, also based on dry weight, were greater than those reported by Kleiber *et al.*

Investigations in this laboratory have indicated that rat mammary tissue contains a very large proportion of fat which may be varied by such factors as pregnancy, lactation, and weaning. It was further noted by use of a histological technique that very little of the activity due to the cytochrome system is associated with the fatty material of the glands, and that almost all the oxygen uptake associated with this system is concentrated in the epithelial cells. In view of these findings, we decided to investigate the Q_{O_2} levels of rat mammary tissue on a water-free, fat-free basis. The endogenous respiration of this tissue was generally low. Excess *p*-phenylenediamine was added to the reaction vessels in order to saturate the cytochrome system. It was considered that, by comparing the endogenous oxygen uptake with the

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Contribution from the Department of Biochemistry and the Department of Anatomy, University of Alberta, Edmonton, Alta. The investigation was supported by a grant from the National Cancer Institute of Canada.

oxygen consumption following the addition of *p*-phenylenediamine, a more accurate measurement could be obtained of the cytochrome reserves in rat mammary tissue.

Experimental

I. Manometric Methods

Male and female adult albino rats of the Wistar strain were used. The animals were killed by decapitation and the required tissues were removed at once and placed in ice-cold calcium-free Ringer's solution (pH 7.4). Inguinal glands were always used, since they can be completely separated from muscle tissue.

The preparation of mammary tissue for measurement of oxygen uptake was a difficult problem. The use of a homogenate was attempted and abandoned because the large amount of fatty material present permitted some of the brei to creep up the walls of the center well of the reaction vessel until it eventually came in contact with concentrated base. We found, as have other workers, that slicing techniques are extremely difficult to apply to mammary glands. It was decided to use scissors to cut several small pieces from approximately the same portion of the gland in each case. Although the tissue was not in pieces as thin as those advocated by Folley and French, the technique did permit rapid transfer from the animals to the reaction vessel, and we found replicates to be in good agreement. Moreover, *p*-phenylenediamine was added in all determinations, and it is known to penetrate readily the fatty material of the gland.

The pieces of tissue were transferred to Warburg reaction flasks, each of which contained 1.8 ml. calcium-free Ringer–phosphate–glucose solution. The center well of each flask contained 0.2 ml. 20% potassium hydroxide and a piece of fluted filter paper. The use of *p*-phenylenediamine as a substrate for the cytochrome system necessitated the use of a control flask, identical with the respiring flask except for the omission of tissue and potassium hydroxide, in order to allow correction for autoxidation. The side arms of control and experimental flasks contained 0.3 ml. of a freshly prepared solution of *p*-phenylenediamine which had been adjusted to pH 7.4 with 0.1 *N* hydrochloric acid. The final concentration of *p*-phenylenediamine in the reaction flasks was 0.2% or 1.85×10^{-2} *M*, since this amount of the substrate has been found sufficient to saturate the cytochrome system in mammary tissue under all experimental conditions studied by us. The vessels were gassed for 10 min. with 100% oxygen, in conformity with the work of Folley and French, placed in a water bath at 37° C., and shaken for 15 min. to bring their contents to equilibrium. The manometers were then read for the next 30 min., by which time a steady rate of endogenous oxygen utilization was measured. At this point *p*-phenylenediamine was tipped in from the side arm of each flask. The first five minute reading following the addition was disregarded and then the manometers were read at five minute

intervals for 20 min. usually, during which time the rate of oxygen consumption was linear. Q_{O_2} and $Q_P^{O_2}$ values for mammary tissue were calculated on the basis of constant oxygen consumption for three consecutive five minute readings. Estimation of the activity of each gland was always done in duplicate.

At the conclusion of all experiments, the pieces of tissue from each vessel were transferred to a Gooch crucible, which had a watch glass set underneath it. The watch glass served to retain any fatty material which separated from the tissue during the overnight drying period. After the material had been dried in an oven at 110° C., it was weighed. It is possible that there may be a slight error in this weight in the case of lactating glands due to the milk error mentioned by Folley and French. However, the tissue was in small pieces, and we believe that most of the milk was leached out in the reaction vessel during the experiment. After the dry weight was determined the tissue was heated with ether in a beaker to remove the bulk of the fat. Finally the remaining fat was removed in a Soxhlet apparatus and the weight of the dried, fat-free tissue was then determined. The values of Q_{O_2} in μl./mgm./hr. were calculated on the basis of (*a*) dry weight (as usually reported in the literature); (*b*) dry, fat-free weight; and, (*c*) dry, fat-free weight following the addition to the reaction vessel of *p*-phenylenediamine.

Results

Female Rat Mammary Tissue

Oxygen consumption was measured in the mammary tissue obtained from adult female rats in various experimental conditions. The values of Q_{O_2} determined on the three bases listed above are presented in Table I.

TABLE I

OXYGEN CONSUMPTION (Q_{O_2}) AND RESPONSE TO *p*-PHENYLENEDIAMINE ($Q_P^{O_2}$) OF MAMMARY TISSUE OF ADULT FEMALE RATS IN VARIOUS EXPERIMENTAL STATES

(Numerical values are averaged and in Columns (*b*), (*c*), and (*d*) the standard error of the mean is indicated)

Experimental state	Number of rats	(*a*) Q_{O_2} on dry weight basis	Fat as % dry weight	(*b*) Q_{O_2} on fat-free dry basis	(*c*) $Q_P^{O_2}$ on fat-free dry basis	(*d*) Increase of (*c*) over (*b*)*
Normal	11	0.9	87	3.7 ± 0.3	9.6 ± 0.78	5.9 ± 0.8
Pregnant (10–14 days)	4	1.8	84	10.2 ± 0.1	17.7 ± 1.28	7.5 ± 1.3
Lactating (18–21 days)	6	5.2	47	10.1 ± 0.26	13.0 ± 0.42	2.9 ± 0.4
Seven days after weaning	2	0.9	83	5.1 ± 0.12	14.8 ± 0.72	
Castrated	4	0.2	95	3.9 ± 0.43	11.5 ± 0.56	

* *The results were analyzed statistically by the method for the significance of the difference of means of small samples. (R. A. Fisher, Statistical methods for research workers. 9th ed. Oliver and Boyd, Ltd., Edinburgh and London. 1944) It was found that the increases in column (d) were highly significant. The increases in Q_{O_2} (b) and $Q_P^{O_2}$ (c) for pregnant and lactating animals, as compared with the corresponding normal values, were also found to be significant.*

Kleiber *et al.* found a threefold increase, on a dry-weight basis of Q_{O_2} of mammary tissue of lactating rats compared with the pregnant animals, and our results are in agreement with theirs. An eightfold increase is reported by Folley and French. Table I indicates that if the Q_{O_2} values are estimated on a fat-free basis (Column *b*) there is a threefold increase over normal in pregnancy, and this is maintained at a constant level during the latter part of lactation, at least. There is a return towards normal values seven days after weaning, which, in this laboratory, occurs 21 days after the birth of the young.

The mammary glands of castrated female rats contain more than the normal amount of fat and the tissue has as a result an abnormally low dry-weight Q_{O_2}.

The very marked enhancement of oxygen uptake which followed the additon of *p*-phenylenediamine indicates, according to Craig, Basset, and Salter (3) that there are in normal, or resting, mammary tissue considerable reserves of the cytochrome system. As indicated in the last column of Table I there is a small but significant rise of these reserves during mid-pregnancy followed by a pronounced lowering during lactation when there are special demands for energy in the glands.

Male Rat Mammary Tissue

Since it was noted histologically that the male rat has a fairly well developed mammary gland, determinations were run on the male mammary tissue. For five adult male rats we found that the average fat content (90%) and $Q_P^{O_2}$ (10.8 ± 0.55) were practically identical with corresponding values for adult female mammary tissue.

II. Histological Methods

During the oxygen consumption studies reported above, it was noticed that when *p*-phenylenediamine was added to the reaction vessels the mammary gland slices appeared to darken only in certain well defined areas. It seemed probable that the color development was associated with oxidation of *p*-phenylenediamine by the cytochrome system and, therefore, that the actual site of oxidation might be determined histologically.

Mammary tissue was obtained from normal adult female rats in the usual way. Small pieces were placed in a test tube and shaken for an hour with 1.8 ml. Ringer–phosphate–glucose solution and 0.3 ml. *p*-phenylenediamine solution, prepared as above for manometric experiments. The tissue was left in the reaction mixture overnight, and in the morning it was washed with distilled water and then treated with 0.1 *N* hydrochloric acid for at least 15 min. to leach out the unoxidized *p*-phenylenediamine. The stained pieces of mammary gland were then blotted dry and cleared overnight in oil of origanum. They were further cleared in xylol and mounted in piccolyte.

As a control, pieces of the same gland were treated with potassium cyanide in order to inhibit oxidation through the cytochrome system. The tissue

PLATE I

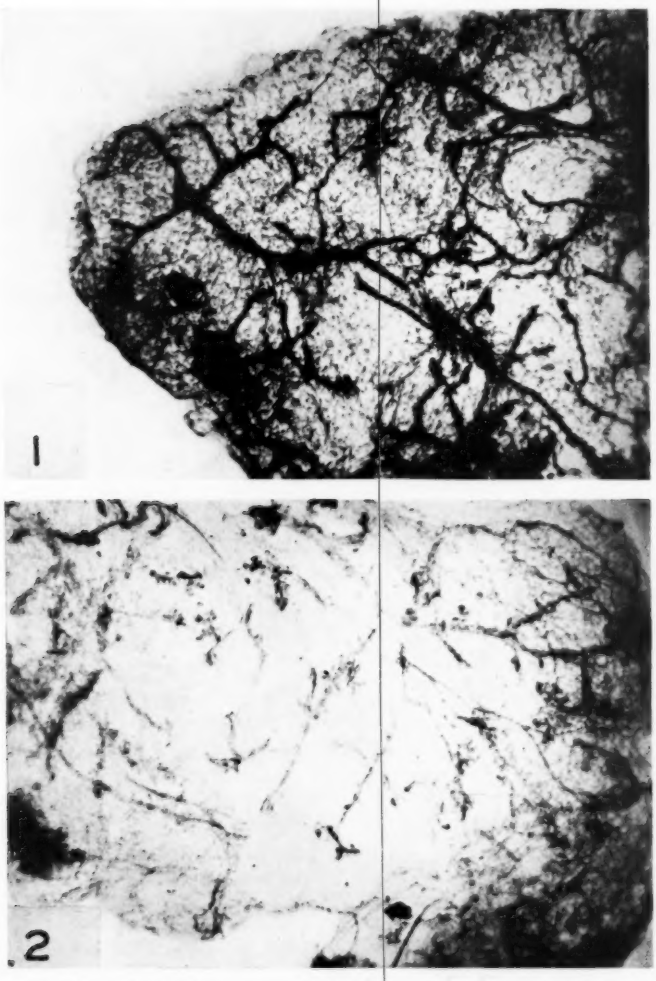

FIG. 1. *Section of inguinal mammary gland of adult female rat, stained with p-phenylenediamine, ×25. The epithelial gland tree is outlined by concentration of the oxidized p-phenylenediamine.*

FIG. 2. *Section of gland used for Fig. 1, ×25. Treatment as with the tissue in Fig. 1 did not show up gland tree because the potassium cyanide prevented oxidation of p-phenylenediamine, but as the figure shows, subsequent staining by hematoxylin revealed that mammary epithelium was present.*

was immersed in 1.5 ml. Ringer–phosphate–glucose solution and 0.3 ml. 0.3 *M* potassium cyanide for 30 min. before the additon of 0.3 ml. *p*-phenylenediamine. The pieces of gland were then treated exactly as the tissue in the active tube.

Results

Figs. 1 and 2 are photomicrographs of the *p*-phenylenediamine stained and the cyanide treated tissues respectively.

The staining seen in Fig. 1 is due to the oxidation of *p*-phenylenediamine and it is clear that the reaction is concentrated in the epithelial cells of mammary gland, occurring only to an insignificant degree in the fat. Some pieces of tissue were treated with potassium cyanide as outlined above and when these were examined histologically no precipitate was found in the epithelial cells. To make sure that mammary gland tissue was present these pieces were subsequently stained in whole mounts by hematoxylin and the presence of a gland tree was established (Fig. 2). This shows that the catalytic mechanism involved in the oxidative response to *p*-phenylenediamine is cyanide sensitive. Various workers, including Commoner (2), consider that cyanide inhibition of tissue oxidation is associated with the cytochrome system. The histological results would justify the assumption that the staining by *p*-phenylenediamine is mediated through the iron-containing cyanide-sensitive cytochrome system in mammary gland epithelium.

Others have reported on stains for the cytochrome system. Montagna and Noback (6, 7), used the nadi reagent (α-naphthol and dimethyl-*p*-phenylenediamine) to stain cytochrome oxidase in mast cells. Becker (1) also used the nadi reaction but stated that it did not allow any conclusion as to the localization of the cytochrome system.

Because of the diluting effect of fat, which we have shown by staining to contain practically no cytochrome, it is felt that values of Q_{O_2}, as reported in this paper, give a precise indication of the respiratory activity of mammary tissue.

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THE USE OF PRISCOLINE (2-BENZYLIMIDAZOLINE HYDROCHLORIDE) AS A TEST IN OCCLUSIVE ARTERIAL DISEASE[1]

By J. Doupe and R. M. Cherniack

Abstract

In 19 subjects with chronic occlusive arterial disease an intramuscular injection of 75 mgm. Priscoline (2-benzyl-2-imidazoline) was found in most cases to produce a greater rise in toe temperature than did the body warming procedure of Gibbon and Landis. In five subjects the vasodilating property of Priscoline was found to be superior to that of spinal anesthesia. In four cases the results with Priscoline predicted the effect of sympathectomy with greater accuracy than did the body warming test. The superiority of the Priscoline test was ascribed to the presence of denervation sensitivity due to a disturbance of sympathetic innervation occurring in association with occlusive arterial disease.

Introduction

Smithwick (16) has recently drawn attention to a group of patients with occlusive peripheral arterial disease who respond poorly to vasodilatation tests but nevertheless benefit remarkably from sympathectomy. It is obvious, that, for such cases, some new diagnostic test is desirable. The simplest and most reliable tests in modern-day use are the reflex thermal test and spinal anesthesia. A new test should be superior to either of these.

Currently, a number of new drugs are under investigation as to their effect on the peripheral circulation. One of these, Etamon (tetraethylammonium chloride), gave early promise (1, 4) of being a useful adjunct to diagnosis but later reports (2, 6) were disappointing. More recently Priscoline (2-benzylimidazoline hydrochloride) which is adrenolytic and sympatholytic (10, 11) has been used as a vasodilating agent in the treatment of peripheral vascular disease (9, 13) and its diagnostic usefulness has also been suggested (8). The present study was therefore undertaken to assess its diagnostic value by comparing its action to the effects of the reflex thermal test, spinal anesthesia, and sympathectomy.

Subjects and Methods

The subjects were the 19 patients shown in Table I, all of whom were referred with a diagnosis of organic occlusive arterial disease of the lower extremities but were otherwise unselected except that they had to be available for two or more tests. Note was made of the presence or absence in the feet of a disturbance of the appreciation of light touch and pin prick. Those cases in which the examiner had difficulty in estimating sensory loss were classified as questionable.

Skin temperature was used as the index of blood flow and was measured by means of copper constantan (gauge 34) thermocouples held in contact with the pads of exposed digits by narrow strips of adhesive tape on the dorsum of the second phalanx. Temperatures were recorded every two to four minutes

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Contribution from the Department of Medicine and the Department of Physiology and Medical Research of The University of Manitoba and from the Winnipeg General Hospital, Winnipeg, Canada.

from two digits of each foot and from the right index finger, and read to 0.1° C. Rectal temperature was recorded by a thermocouple and read to 0.01° C. Room temperature was kept constant at 19° – 21° C. and measured by a thermocouple suspended several feet above the parts under observation.

Subjects reclined in the constant temperature room clad only in a hospital gown. To provide a base line, recordings were made for 10 to 30 min. before a test. The reflex thermal test of Gibbon and Landis (7) was performed by immersing the left arm in water at 44° C., and by covering the trunk and proximal parts of the extremities with blankets. When, after prolonged heating, a definite plateau of maximum skin temperature was attained in the toes and the control finger, the blankets were removed and the left arm placed in cold water at 18° C. This stimulated reflex vasoconstriction, thereby testing the integrity of the sympathetic nerves. On another day, each subject received 75 mgm. Priscoline intramuscularly, and the effect was observed for one and one-half to two hours. Blood pressure, recorded at five minute intervals, was little affected. Five of the subjects received 100 mgm. Novocaine intrathecally. In all, analgesia was produced at least to the level of the 10th thoracic dermatome and the response was observed for one hour or longer. The effect of lumbar sympathectomy on skin temperature was observed in four of the subjects two to four weeks after operation at which time the reflex thermal test was repeated.

Results

During each procedure note was taken of the height to which the skin temperature rose. A comparison of the maximum temperatures attained during the reflex thermal test and the Priscoline test is shown in Fig. 1 and Table I.

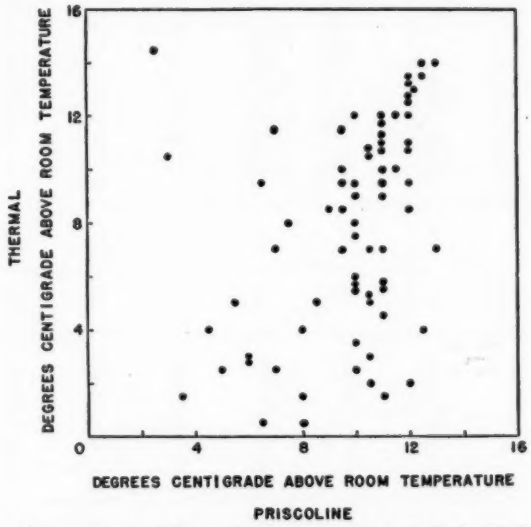

FIG. 1. *A comparison of the maximum temperatures attained with the reflex thermal test and the intramuscular injection of 75 mgm. of Priscoline.*

TABLE I

SUBJECTS AND RESULTS

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Maximum temperature, °C.		
								Thermal	Priscoline	Difference
1	Arteriosclerosis	58	M	Pain, numbness left leg for 2 years. Pulsations: L−, R+	LT4	N	N	31.5	24.0	− 7.5
					LT1	N	N	30.5	27.5	− 3.0
					RT4	N	N	34.5	33.0	− 1.5
					RT1	N	N	34.5	33.0	− 1.5
2	Arteriosclerosis	51	M	Pain in both calves and ankles for 4 years. Pulsations: L+, R+	LT4	N	N	31.0	32.0	+ 1.0
					LT1	N	N	31.5	31.5	0
					RT4	N	N	32.0	32.0	0
					RT1	N	N	30.5	31.0	+ 0.5
3	Arteriosclerosis	75	M	Pain in both calves, worse in left, for 1 year. Pulsations: L−, R−	LT4	N	N	26.0	31.5	+ 5.5
					LT1	N	N	25.5	32.0	+ 6.5
					RT4	N	N	32.0	33.0	+ 1.0
					RT1	N	N	32.0	33.0	+ 1.0
4	Arteriosclerosis	61	M	Pain in both calves, worse in left, for 3 years. Pulsations: L−, R−	LT5	?	N	30.5	33.0	+ 2.5
					LT1	?	N	31.0	32.5	+ 1.5
					RT5	N	N	35.0	34.0	− 1.0
					RT1	N	N	34.5	33.5	− 1.0
5	Arteriosclerosis	66	M	Pain in both calves, worse in left, for 4 years. Pulsations: L−, R−	LT4	?	N	24.0	30.5	+ 6.5
					LT1	?	N	23.5	31.0	+ 7.5
					RT4	?	N	33.5	33.0	− 0.5
					RT1	?	N	33.0	32.5	− 0.5
6	Arteriosclerosis	69	M	Duodenal ulcer. Pain in right calf and cold feet for 2 years. Pulsations: L−, R−	LT4	A	N	33.5	33.0	− 0.5
					LT1	A	N	34.0	33.0	− 1.0
					RT4	A	N	33.0	32.0	− 1.0
					RT1	A	N	30.0	32.0	+ 2.0
7	Arteriosclerosis	63	M	Pain and numbness left leg for 3 years. Burning left sole for 1 year. Pulsations: L−, R−	LT4	?	A	26.0	31.5	+ 5.5
					LT1	?	A	25.0	29.0	+ 4.0
					RT4	?	N	30.5	32.5	+ 2.0
					RT1	?	N	29.5	30.5	+ 1.0

* *LT4 means left fourth toe, RT1, right first toe, etc.*

** *N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.*

TABLE I—*Continued*

SUBJECTS AND RESULTS—*Continued*

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Maximum temperature, °C.		
								Thermal	Priscoline	Difference
8	Arteriosclerosis	64	M	Pain in both calves, worse in left, for 4 years. Pulsations: L−, R−	LT4	?	A	26.5	31.0	+ 4.5
					LT1	?	A	24.5	31.0	+ 6.5
					RT4	?	A	28.0	32.0	+ 4.0
					RT1	?	A	26.5	32.0	+ 5.5
9	Arteriosclerosis	69	M	Pain in both calves for 3 years. Numbness and cold feet—5 months. Pulsations: L+, R+	LT4	A	A	23.0	33.0	+10.0
					LT1	A	A	22.5	32.0	+ 9.5
					RT4	A	N	28.0	34.0	+ 6.0
					RT1	A	N	25.0	33.5	+ 8.5
10	Buerger's	40	M	Amputated right leg 2 years. Numbness and pain in left leg for 9 months. Pulsations: L−	LT4	N	N	31.5	31.5	0
					LT1	N	N	29.5	33.0	+ 3.5
11	Buerger's	40	M	Pain in left great toe for 7 months. Pulsations: L−, R−	LT4	N	N	25.5	25.0	− 0.5
					LT1	N	N	26.0	26.5	+ 0.5
					RT4	N	N	32.5	28.0	− 4.5
					RT1	N	N	35.5	23.5	−12.0
12	Buerger's	30	M	Pain in right thigh and calf on walking for 3 months. Slight numbness. Pulsations: L+, R−	LT5	N	N	35.0	33.5	− 1.5
					LT1	N	N	33.0	33.0	0
					RT5	N	N	22.5	29.0	+ 6.5
					RT1	N	N	23.5	28.0	+ 4.5
13	Buerger's	47	M	Pain in left calf and right foot, numb cold feet for 1 year. Pulsations: L−, R−	LT4	?	A	33.0	32.0	− 1.0
					LT1	?	A	32.0	32.0	0
					RT4	?	A	32.5	30.5	− 2.0
					RT1	?	A	31.0	30.5	− 0.5
14	Buerger's	50	M	Pain in both legs and right great toe; worse at night. Pulsations: L−, R−	LT4	A	A	32.0	32.0	0
					LT1	A	A	30.0	31.0	+ 1.0
					RT3	A	A	24.0	27.0	+ 3.0
					RT1	A	A	24.0	27.0	+ 3.0

* *LT4 means left fourth toe, RT1, right first toe, etc.*

** *N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.*

TABLE I—*Concluded*

SUBJECTS AND RESULTS—*Concluded*

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Maximum temperature, °C.		
								Thermal	Priscoline	Difference
15	Diabetes Arteriosclerosis	59	M	Pain in both calves, particularly right for 9 years. Cold and numbness, particularly right leg—3 years. Pulsations: L−, R−	LT4	A	A	28.0	30.5	+ 2.5
					LT1	A	A	29.5	30.0	+ 0.5
					RT4	A	A	28.0	31.5	+ 3.5
					RT1	A	A	33.0	31.0	− 2.0
16	Diabetes Arteriosclerosis	63	F	Numbness and cold feet for 10 years. Ulcers both ankles for 9 months. Pulsations: L−, R−	LT4	A	A	23.0	31.5	+ 8.5
					LT1	A	N	26.5	32.0	+ 5.5
					RT4	A	A	21.5	27.5	+ 6.0
					RT1	A	A	21.5	29.0	+ 7.5
17	Diabetes Arteriosclerosis	66	F	Pain, numbness, and cold feet for 3 years. Pulsations: L−, R−	LT4	N	N	31.0	30.5	−0.5
					LT1	A	A	26.0	29.5	+ 3.5
					RT4	A	A	23.5	26.0	+ 2.5
					RT1	A	A	22.5	24.5	+ 2.0
18	Diabetes Arteriosclerosis	48	F	Amputated right leg, 4 years. Numbness left leg, 1 year. Gangrene left great toe. Pulsations: L−	LT4	A	A	29.0	28.5	− 0.5
					LT1	A	A	28.0	28.0	0
19	Diabetes Arteriosclerosis	57	M	Burning feeling in both legs and feet for 2 years. Pulsations: L−, R−	LT4	?	A	28.5	31.0	+ 2.5
					LT1	?	A	27.0	31.0	+ 4.0
					RT4	?	A	27.0	31.0	+ 4.0
					RT1	?	A	29.0	31.0	+ 2.0

* *LT4 means left fourth toe, RT1, right first toe, etc.*

** *N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.*

It will be seen that 44 of the 72 digits attained a higher temperature with Priscoline than with reflex heating. In 31 of these the difference was greater than 2° C. In only four digits did the effect of the reflex thermal test exceed that of the Priscoline by more than 2° C.

Fig. 2 shows that in the 20 digits tested the effect of Priscoline was greater than that of reflex heating or spinal anesthesia.

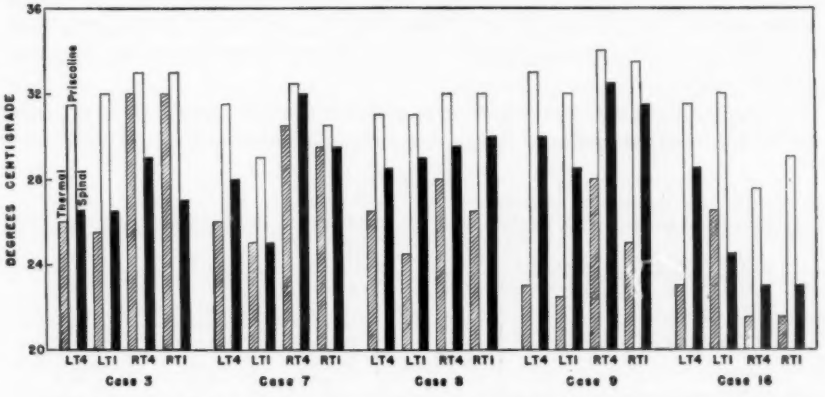

FIG. 2. *A comparison of the maximum temperatures attained in toes with the reflex thermal test, intramuscular Priscoline, and spinal anesthesia.*

The preoperative and postoperative results in the four subjects who were sympathectomized are shown in Table II. The average error of the reflex thermal test in the prediction of the operative result was 4.2° C. while with Priscoline it was 2.2° C.

TABLE II

THE EFFECT OF PREOPERATIVE TESTS AND SYMPATHECTOMY ON SKIN TEMPERATURE (TEMPERATURES GIVEN IN °C. ABOVE ROOM TEMPERATURE)

Case No.	Digit	Thermal	Priscoline	Sympathectomy
5	LT4	3.0	9.5	5.5
	LT1	2.5	10.0	8.0
10	LT4	10.5	10.5	12.0
	LT1	8.5	12.0	12.5
12	RT5	1.5	8.0	10.5
	RT1	2.5	7.0	9.0
18	LT4	8.0	7.5	10.0
	LT1	7.0	7.0	10.0

During the course of the experiments it was observed in certain cases that the vasomotor behavior of the digits was abnormal. This abnormality ordinarily consisted of a failure of the vessels of certain toes to constrict in response

to a general cold stimulus although the vessels of the control finger and other toes showed a good vasoconstrictor response. In a smaller number of cases, the vasomotor behavior was judged to be abnormal because there was an absolute absence of any vasodilatation in response to prolonged heating. That the failure to increase the blood flow was not due to arterial disorder in this group of cases was shown by the ability of other procedures such as Priscoline to produce vasodilatation. These abnormal vascular reactions resembled those reported by Doupe (5) and Richards (12) in individuals with peripheral nerve lesions. This and the abnormalities of sensation suggested the presence of a neuropathy.

It was therefore of interest to observe whether the efficacy of Priscoline could be related to any evidence of this postulated nerve lesion. Table III indicates

TABLE III

THE EFFECT OF DENERVATION ON THE RELATIVE EFFICACY OF THE INJECTION OF PRISCOLINE AND THE REFLEX THERMAL TEST

Evidence of denervation	Total number of digits	Number of digits		
		Priscoline less than reflex	Priscoline equal to reflex	Priscoline greater than reflex
No sensory loss	25	4	16	5
Questionable sensory loss	22	0	10	12
Definite sensory loss	25	0	11	14
Normal vasomotor behavior	40	4	25	11
Abnormal vasomotor behavior	32	0	12	20

that, in comparison to the cases with normal innervation, those with sensory loss or abnormal vasomotor behavior had a higher proportion of digits in which the response to Priscoline was greater than the response to the reflex thermal test. Results within 2° C. of each other were considered equal.

Discussion

It is apparent from the above results that in cases of clinical peripheral occlusive arterial disease Priscoline had a greater vasodilating effect than either reflex heating or spinal anesthesia. In a limited number of cases it was further found, in agreement with Smithwick (16) that the reflex thermal test tended to underestimate the effect of sympathectomy. In contrast to this the results of the injection of Priscoline approximated those obtained by sympathectomy.

The explanation for the superiority of Priscoline in certain cases may depend on the existence of a state of hypersensitivity to adrenaline. In the first place it has been established by Cannon (3) that sympathetic denervation greatly increases the sensitivity of vessels to adrenaline and secondly there is evidence that a peripheral nerve lesion exists in many cases of occlusive arterial disease. Thus Shumacker (15) noted a defect in vasoconstrictor ability in cases of

arteriosclerosis and Buerger's disease, while Rundle (14) has reported a similar condition associated with minimal sensory changes in diabetics. In the present series similar evidence indicative of a peripheral nerve lesion was found. Therefore it is not surprising that Priscoline, being adrenolytic as well as sympatholytic was more potent than either sympathetic inhibition or block.

It appears paradoxical that sympathectomy should increase the blood flow to digits already denervated, though this has been shown to occur in cases of proven traumatic nerve lesions (5). The explanation for this may be that sympathectomy prevents the liberation of adrenaline from endings adjacent to those which are hypersensitive. In addition it is possible that the increased warmth of the whole extremity impedes the vasoconstricting action of a cold environment to which peripherally denervated vessels are also hypersensitive (5).

These considerations suggest that the resemblance between the effects of Priscoline and sympathectomy, though real and valuable, is nevertheless fortuitous. It appears that most vasodilatation tests are unsatisfactory because they are likely to induce an increased secretion of adrenaline and because their short duration does not permit the spasm due to the cold environment to be overcome. For these reasons tests which depend simply on blocking or inhibiting vasoconstrictor impulses do not necessarily give results corresponding to those obtained by sympathectomy. On the other hand, if part of the effect of Priscoline is attributable to its adrenolytic property, it too would not necessarily duplicate the effects of sympathectomy. Nevertheless the present results suggest that it does this more closely than do the other procedures.

The results reported here strongly support the contention of Green and Ogle (8) that Priscoline is useful in distinguishing between the functional and organic elements in occlusive arterial disease. This appears to be particularly true where the spasm is secondary to denervation sensitivity.

Acknowledgments

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THE HAEMOGLOBINS OF THE FOETUS AND NEWBORN[1]

By F. D. White, G. E. Delory, and L. G. Israels[2]

Abstract

In addition to the two forms of haemoglobin previously recognized in the blood of the foetus and newborn, a third form has been detected by following the rate of reaction with sodium hydroxide. This component appeared in 33 of the 66 samples studied but could not be detected in the remainder. No explanation could be found for this anomaly. Confirmation was obtained of the presence of a second form of haemoglobin in adult blood. This was shown to differ from the most refractory fraction of foetal blood. It is suggested that the older terminology is no longer sufficiently definitive and an alternative scheme is proposed.

Introduction

It has been known for many years that the haemoglobin of human blood can exist in at least two forms. One of these forms, "foetal" or "refractory" haemoglobin, occurs mainly in the blood of the foetus and newborn infant; while the other, "adult", "labile", or "later" haemoglobin, is present in the adult and in children over four months of age (10).

The two haemoglobins have been shown to differ in many ways, notably in their oxygen dissociation curves (2, 12, 7), the rate of spread of their monomolecular films (3), their antigenicity (5), their amino acid composition (14), and in their reaction with alkali (15). The corresponding methaemoglobins also differ in regard to solubility and crystalline structure (11). It is now generally accepted on the grounds of evidence derived from these procedures, together with the investigations of Haurowitz (8, 9), that the differences in these haemoglobins reside in the globin portion of the molecule—the ferroporphyrin group being identical.

Following the qualitative studies of Von Kruger, Haurowitz determined the relative concentrations of these haemoglobins in bloods containing both forms, by utilizing the difference in their rates of conversion into alkaline globin haematin. This principle was modified and extended by Brinkman and Jonxis (3), Baar and Lloyd (1), and recently by Ponder and Levine (13).

In the course of a study carried out in this Department of the part played by foetal haemoglobin in the aetiology of neonatal jaundice, some apparently unreported observations were made which seem worthy of record.

Experimental

Note on Terminology Used

In view of the lack of a universally accepted nomenclature for the haem pigments, it should be pointed out that we have called the ultimate product obtained by treating haemoglobin with sodium hydroxide, alkaline globin

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haematin. This reaction involves the oxidation of the haem iron to the ferric state and the denaturation of the globin moiety. For simplicity of presentation, references to the change from haemoglobin to alkaline globin haematin will be referred to as "denaturation".

Method

The method, which is an adaptation of that of Baar and Lloyd (1), is based on the fact that, if the rate of conversion of haemoglobin to alkaline globin haematin depends on the amount of unchanged haemoglobin present at any time, then,

$$\log (H_t/H_0) = -kt$$

where H_0 and H_t are the haemoglobin concentrations initially and at time t respectively, and k is the reaction constant.

The procedure used was as follows: A sample of blood was centrifuged, the plasma pipetted off, and the red cells washed twice with normal saline; 0.5 — 1.0 ml. of the red cells was introduced into approximately 100 ml. of 0.1% sodium carbonate solution. The thoroughly mixed solution was oxygenated in a Waring Blendor, filtered, and its optical density measured in a Beckman Spectrophotometer. More sodium carbonate solution was then added, if necessary, to bring the optical density to a convenient value (0.90 — 1.20).

Two-milliliter samples of this solution were pipetted accurately into each of two cuvettes, to the first of which 0.4 ml. of water was added using a 1 ml. tuberculin syringe. The optical density of this solution (D_{H_0}) was a measure of the initial haemoglobin concentration.

To the second cuvette, 0.4 ml. of $N/4$ sodium hydroxide solution was added in a similar manner to facilitate rapid manipulation and the cuvette shaken to ensure adequate mixing. A stop watch was started at the moment of sodium hydroxide addition. The cuvette was placed immediately in the spectrophotometer and the optical density read as frequently as possible for 10 min. The readings may be represented as D_{H_t}.

At the completion of these readings, the test solution was allowed to stand for 24 hr. and its optical density D_B taken again. The calculations were made as follows:

$$\frac{H_t}{H_0} = \frac{D_{H_t} - D_B}{D_{H_0} - D_B}$$

By plotting the logarithmic ratio of the haemoglobin concentrations at the time t and initially, against the time in minutes, and extrapolating to zero time (Fig. 1), the percentages of the various haemoglobin fractions were obtained.

Preliminary experiments showed that the greatest difference in optical density between oxyhaemoglobin and alkaline globin haematin occurs at a wave length of 578mμ and our measurements were therefore made at this wave length. Baar and Lloyd used the Evelyn colorimeter with a 540 filter

but the use of the Beckman Spectrophotometer (model DU), which allows precise selection of a narrow spectral band, made it possible to obtain a sharper separation of the two pigments.

To minimize errors which might be introduced by icteric plasma, red cells centrifuged free from plasma were used throughout.

All determinations were carried out in duplicate.

Preparation of a Partially Purified Sample of "Refractory" Haemoglobin

A partially purified sample of the most refractory fraction of foetal haemoglobin was prepared so that its denaturation curve could be compared with those of cord blood and adult blood. The preparation was carried out as follows:

To 50 ml. of a 10% solution of cord blood in 0.1% sodium carbonate solution, 10 ml. of normal sodium hydroxide was added. After being allowed to stand for five minutes at room temperature, the solution was buffered by the addition of 20 ml. of normal sodium bicarbonate solution. The denatured globin fraction was then precipitated out by the addition of 80 ml. of a saturated solution of sodium sulphate. This was filtered and the clear bright red filtrate was found to contain only the most refractory fraction of foetal blood, as shown by study of its denaturation curve (Fig. 1) and the nonappearance of the characteristic band from alkaline globin haematin when examined in the Hartridge Reversion Spectroscope after the addition of ammonium hydroxide and ethyl alcohol. This was in contrast to the precipitated pigment, which, when taken up in alcohol and ammonium hydroxide, clearly showed the band in question.

Results

Fig. 1 shows typical denaturation curves for adult blood, cord blood, and a partially purified preparation of the most refractory component of foetal blood. While the latter takes the form of a straight line, the curve for adult blood appears as two straight lines. Since the slope of the line on the graph is a measure of the reaction constant, it may be assumed that adult blood consists of at least two forms of haemoglobin.

The situation is different, however, with cord blood (see Fig. 2 in which this curve is shown on a larger scale). In the typical example given, there is a steep slope to the one minute level; a gradual slope from the one to the three minute level, and a still more gradual slope from three minutes onwards. This would suggest that in this particular case three haemoglobins were present.

The terms "adult", "labile" or "later" haemoglobin, and "foetal" or "refractory" haemoglobin were introduced when it was believed that only two forms of haemoglobin existed. With the development of our knowledge, it is felt that these terms are not sufficiently definitive, and it is recommended that the haemoglobins of adult blood be designated a_1 and a_2, and those of

foetal blood f_1, f_2, and f_3 (see Figs. 1 and 2). Of the components of foetal blood the fractions now designated f_1 and f_3 have been known for some time. Here we are concerned with the newly described form f_2.

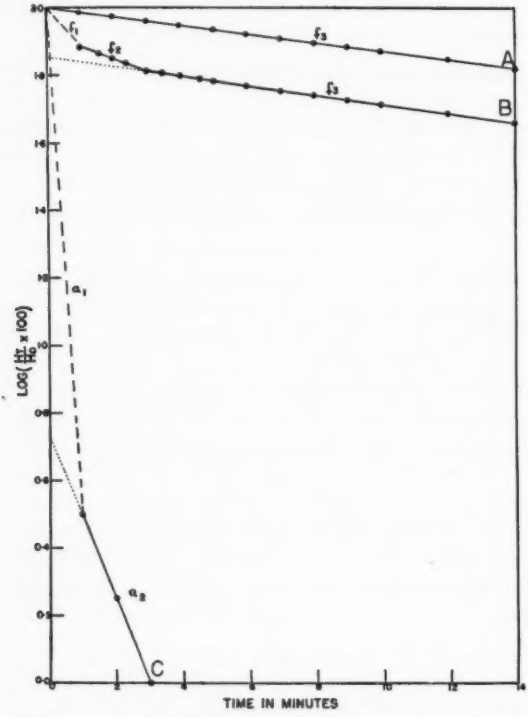

FIG. 1. *Typical curves showing rate of "denaturation" of:— A, partially purified refractory haemoglobin; B, cord blood; and C, adult blood.*

This form was not, however, a constant finding in our series. Of the 66 samples of infant and cord blood examined, it could be detected in only 33 cases. In the samples studied, blood was collected by syringe from the umbilical cord and by heel puncture from newborns at one, three, and seven days and again at three or four weeks after birth. All the babies were normal newborns delivered at the Winnipeg General Hospital at or near full term.

Table I shows the number of samples of cord and newborn blood at the various ages in which this third form of haemoglobin (f_2) was apparent. It is seen from this table that it does not occur more frequently at any particular age, nor did those cases showing this third form appear to differ in any other way from those in which it was not detected.

Further light might be thrown on this problem by measurement of the reaction constants. In view, however, of the dependence of this constant on the temperature, it would be necessary to carry out all measurements at the

same constant temperature. Unfortunately, a constant temperature room or other suitable thermostatic device was not available and attempts to vary the temperature in a controlled manner led to delay in the obtaining of readings

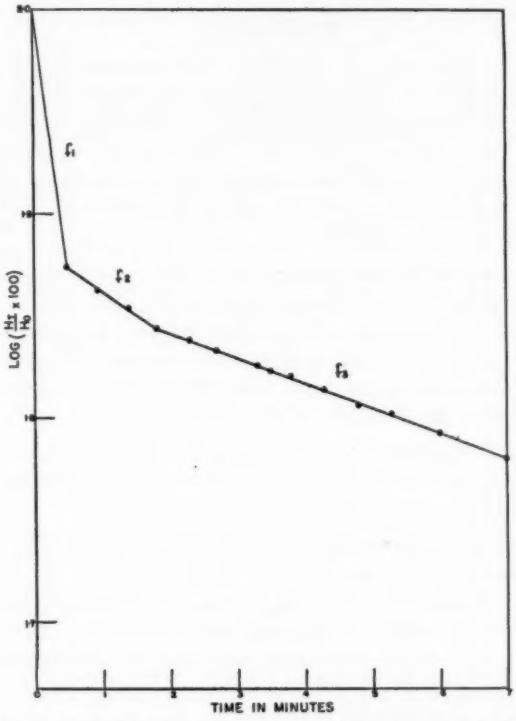

FIG. 2. *Curve B, Fig. 1, drawn on a larger scale to show more clearly the distinct breaks.*

TABLE I

INCIDENCE OF THE APPEARANCE OF THE INTERMEDIATE FORM OF HAEMOGLOBIN (f_2) IN 66 CASES OF CORD AND INFANT BLOOD

	No. of cases where intermediate form apparent	No. of cases where intermediate form *not* apparent	Total
Cord blood	11	12	23
Babies' blood (age three days)	12	9	21
Babies' blood (age seven days)	7	11	18
Babies' blood (age three weeks)	3	1	4
	33	33	66

which was fatal to our purpose. It was found, however, that once the spectrophotometer had been allowed to warm up, the temperature of the solution to be examined remained constant throughout the course of the experiment.

Discussion

The presence of three distinct breaks in the denaturation curves for the blood of the foetus and newborn suggests that haemoglobin may exist here in yet a third form. This form (designated f_2) is additional to those previously described (labile, f_1, and refractory, f_3). This new form, which is characterized by a denaturation rate intermediate between those of the other two forms, could not be detected in all of the samples studied; nor could its presence be correlated with any other factor.

So far as we are aware, the demonstration by denaturation experiments of more than two forms of haemoglobin in the blood of the foetus and newborn has not been recorded hitherto. Brinkman and Jonxis disregarded the one and two minute readings in their published curves while Ponder and Levine who were only interested in the most refractory fraction (f_3) took their first reading 10 min. after the addition of the alkali.

It may be noted, however, that Derrien and Roche (6) have reported the isolation, by salting out methods, of three different haemoglobins from adult, and five from the blood of the newborn.

Turning now to the consideration of adult blood, the finding of two components of haemoglobin, first described by Brinkman *et al.* (4), has been confirmed by us. The possibility that the refractory components of foetal and adult blood (a_2 and f_3) might be identical was considered. If this were the case, one might expect the slopes of these two forms on the denaturation curve to be identical.

In our experiments, however, this was not the case (see Fig. 1), the refractory fraction of adult blood having a slope about twice that of the most refractory fraction of cord blood.

In summary, then, it is suggested that adult blood contains at least two haemoglobins (a_1 and a_2) and haemoglobin of the foetus and newborn may contain three fractions (f_1, f_2, and f_3), one of which (f_2), however, is not always detectable. It may well be that the fractions a_1 and f_1 are identical as seems to be tacitly assumed by all previous workers, although this assumption still remains to be proved.

The evidence adduced here from the rate of reaction with sodium hydroxide indicates that the refractory component of adult blood (a_2) is not identical with either of the refractory components of foetal blood (f_2 and f_3).

Acknowledgments

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THE ROLE OF THE FOETAL HAEMOGLOBINS IN THE AETIOLOGY OF JAUNDICE OF THE NEWBORN[1]

By G. E. Delory, L. G. Israels,[2] and F. D. White

Abstract

The total bilirubin, total haemoglobin, and haemoglobin fractions were studied in the cord blood and in the heel puncture blood at one, three, and seven days, and at three or four weeks after birth, in 32 normal newborns. No relationship was found to exist between the rate of destruction of the total haemoglobin, or of its most refractory component f_3, and the degree of bilirubinaemia. The proportion of this f_3 component did not always undergo progressive steady destruction, nor did it seem to be selectively destroyed in the neonatal period and it would not appear to play any special role in the production of neonatal jaundice.

Introduction

For over 100 years, the aetiology of jaundice of the newborn has been under investigation without a clear answer forthcoming. Among the hypotheses which have been considered are: (1) increased destruction of haemoglobin due to the hyperhaemoglobinaemia occurring immediately after birth and (2) decreased elimination of bilirubin by the "immature" liver. Although only about half of all normal newborns become jaundiced after birth, the bilirubin level is always much higher than in the adult. The fact that the bilirubin concentration required to produce clinical jaundice in the newborn is considerably higher than that in the adult has been noted by many workers (11, 14) and may depend on a difference in capillary permeability or bilirubin binding power of the collagenous and elastic tissues of the skin (14). Waugh *et al.* (10) could find no absolute blood bilirubin level at which jaundice developed in all infants.

A direct relationship is, however, known to exist between the bilirubin level of the cord blood (15), the iron content of the placenta (13), and the degree and duration of the neonatal hyperbilirubinaemia. A comprehensive review of this subject has been published by Weech (11).

It has also been well known since the time of Von Kruger (9) that not only is the haemoglobin content higher in the newborn infant but that a qualitative difference also exists. As a simple illustration, when adult and cord blood are separately treated with sodium hydroxide solution, the former changes rapidly to the brown color of alkaline globin haematin while the latter may remain red for many hours. It is known that adult blood contains at least two haemoglobins and that foetal blood may contain three forms, each differing in its rate of reaction with sodium hydroxide (2, 12).

The predominant form of haemoglobin in the foetus has been loosely called "refractory" or "foetal" haemoglobin; that in the adult "labile", "adult", or "later" haemoglobin. With the increase in our knowledge, these terms are

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not sufficiently definitive and in this paper, we shall call the two pigments of adult blood a_1 and a_2 and those of the foetal and newborn blood f_1, f_2, and f_3. This aspect is fully discussed by us in the preceding paper (12). All that need be said here is that the forms a_1 and f_1 are rapidly changed to alkaline globin haematin by the action of sodium hydroxide while a_2, f_2, and f_3 react much more slowly.

It occurred to us that it would be interesting to study the relationship (if any) between the concentrations of these pigments and the degree of neonatal hyperbilirubinaemia since it seemed possible that the development of jaundice might be due to the fact that one of these haemoglobins is more readily broken down to bilirubin.

Jonxis (5) propounded a somewhat similar hypothesis with respect to erythroblastosis. He claimed that in this condition foetal haemoglobin (f_3) is selectively broken down almost to the exclusion of the least refractory type (f_1). Neither Baar (1) nor Ponder and Levine (7) could confirm these observations.

So far as we know, however, no one has considered the role of the foetal haemoglobins in the aetiology of physiological jaundice.

Methods

The babies studied were normal newborns delivered at or near full term at the Winnipeg General Hospital. Blood was collected by syringe from the umbilical cord and by heel puncture from the newborn at one, three, and seven days and again at three or four weeks, for the determination of total bilirubin, and total and differential haemoglobin. Potassium oxalate was used as the anticoagulant.

Total haemoglobin was determined by measuring the optical density of blood in a 1 in 250 dilution with 0.1% sodium carbonate solution. The measurement was made by spectrophotometric readings and standardized by iron analyses carried out according to the titanous chloride method of Delory (3).

Total bilirubin was determined by the method of Waugh (10) modified for the use of 0.1 ml. of plasma.

The degree of jaundice was assessed by inspection of the infant's skin and oral mucous membranes, and graded as follows: no jaundice −, doubtful + −, slight +, moderate ++, and severe jaundice +++.

Estimation of the haemoglobin fraction (f_3) was carried out by the procedure described in the preceding paper.

A recovery experiment was performed to determine the reliability of the method used to estimate the foetal haemoglobin fraction (f_3). Haemoglobin solutions of adult and cord blood were prepared and their denaturation curves determined. The relative concentrations of the haemoglobin fractions were calculated in the usual manner. These solutions were then mixed in varying proportions, the estimation repeated, and the values obtained compared with the calculated values. The results of a typical experiment as shown in

Table I appear to afford satisfactory evidence of the reliability of the method, since the maximum difference between the experimental and calculated values was 4% of the total haemoglobin.

TABLE I

COMPARISON OF THE EXPERIMENTALLY DETERMINED VALUES OF REFRACTORY HAEMOGLOBIN (f_3) WITH THOSE CALCULATED FROM THE DETERMINED VALUE OF 73 FOR ADULT BLOOD AND ZERO FOR CORD BLOOD

Haemoglobin mixture		Refractory haemoglobin (f_3) as percentage of total haemoglobin	
Solution of:		Experimental value	Expected value
Cord blood	Adult blood		
10 ml.	2 ml.	61.5	61
10 ml.	5 ml.	53	49
10 ml.	10 ml.	36.5	36.5
5 ml.	10 ml.	22	24
2 ml.	10 ml.	13	12
1 ml.	10 ml.	9	7

Results

The bilirubin, total haemoglobin, and refractory haemoglobin content of the blood was estimated in a series of 32 normal newborn infants. In the majority of cases, determinations were carried out on the cord blood, and on the third and seventh days of life, while in some cases values were also obtained at three or four weeks of age.

The results are summarized in Table II, while the complete data, including the varying degrees of jaundice, are reported in the appendix.

TABLE II

BILIRUBIN AND HAEMOGLOBIN CONCENTRATIONS IN CORD BLOOD AND BLOOD OF THE NEWBORN

	Cord blood	Third day	Seventh day	Three weeks	Four weeks
Bilirubin					
No. of cases	30	28	30	6	6
Range (mgm. per 100 ml.)	0.7–3.5	1.1–15.2	0.9–10.0	0.6–2.8	0.6–2.3
Mean (" " " ")	1.5	4.9	4.2	1.4	1.2
Total haemoglobin					
No. of cases	31	31	31	6	6
Range (gm. per 100 ml.)	11.4–20.0	14.6–22.8	14.2–22.5	15.2–20.0	10.5–19.0
Mean (" " " ")	14.9	19.2	18.2	17.3	14.9
Refractory haemoglobin					
No. of cases	32	31	32	6	6
Range (% total Hb)	50–87	45–87	44–87	57–69	40–79.5
Mean (" " ")	76.2	76.1	76.3	64.7	70.2
Mean (gm. per 100 ml.)	11.2	14.4	13.7	11.0	10.8

Discussion

In this series of 32 normal newborns, 19, or 59%, developed a definite clinical jaundice within the first seven days of life (cf. Appendix). This is within the range 50-75% which is generally quoted (Smith (8)) although, of course, figures such as this depend on the individual worker's concept of clinical jaundice. There was no bilirubin level (threshold) at which all infants become jaundiced but jaundice was present in all infants with a plasma bilirubin greater than 4.5 mgm. per 100 ml. The fact that the plasma bilirubin level was at its highest recorded value by the third day in the majority of the cases and had fallen by the seventh day of observation is also in agreement with the findings of other workers (15, 11). Considering now the total haemoglobin results, it will be seen that these values also rose sharply after birth and then began a sharp decline reaching the initial level in three or four weeks. Our findings of 14.9, 19.2, and 18.2 as the mean values for the total haemoglobin of cord blood and blood taken on the third and seventh days respectively, may be compared with the mean values reported by Waugh and his associates (10) of 15.36 for cord blood and 15.46 and 14.7 for the fourth and seventh days of life respectively. Faxin (4) quotes mean values of 23.2 and 21.7 for the first and third days of life, while Smith (8) found mean values of 17.9 for cord blood and 19.0 during the first week. It is clear that there is still no agreement on this important question.

With regard to the values obtained for the f_3 fraction of haemoglobin, the mean value of 76.2% found by us for cord blood is in good agreement with the value of 79.75 reported by Ponder and Levine (7) for their series of 15 normal newborns. The range found by us (50-87%) differs, however, from the findings of Jonxis (6) whose values varied from 75-98%.

The steady decline in the concentration of the most refractory component as described by some authors e.g. Jonxis, was found by us to be an over-simplification of the situation. In our studies, the mean values for this fraction showed a steady increase (paralleling the total haemoglobin) up to the seventh day and then a fall to the third or fourth week at which time there was some evidence that the f_3 fraction was decreasing at a greater rate than were those other components which together make up the total haemoglobin. It must be pointed out, however, that all the cases studied did not show the typical pattern suggested by the mean values.

Since from a study of our recovery experiments and duplicates, 4% of the total haemoglobin may be taken as a significant variation in the consecutive determinations of f_3, then on this basis, only 10 of the 32 infants showed a consistent decline in f_3 after birth. The level in 12 infants remained the same for as long as they were followed; five newborns exhibited a rapid rise immediately after birth and five others showed an initial decline followed by a subsequent rise.

Statistical analysis revealed no correlation between the f_3 content of cord blood, and the degree of bilirubinaemia developed by the infant. The correlation coefficient for this relationship was found to be 0.34 for 23 degrees of

freedom, significance at the 5% point being 0.38. Similarly no significant correlation could be established between the rate of replacement of the f_3 fraction and the degree of bilirubinaemia. The correlation coefficient here was found to be 0.22 for 49 degrees of freedom. Significance at the 5% point is 0.38.

Thus no evidence could be found that the f_3 haemoglobin fraction is more susceptible to destruction than the other fractions and hence it appears to be without special significance in the aetiology of neonatal jaundice.

The possible existence of a relationship between the total haemoglobin level of the blood and its subsequent rise and fall was also considered. Inspection of the results and the construction of scattergrams, however, failed to demonstrate any such relationship. The nature of the scattergrams was such that any attempt at statistical analysis was pointless, and in view of the similar conclusions of Waugh *et al.*, was not carried out.

Acknowledgments

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APPENDIX

COMPLETE DATA OF CASES SUMMARIZED IN TABLE II

Case No.	Sex	Degree of icterus *, **		Bilirubin, mgm./100 ml.				Total haemoglobin, gm./100 ml.				Refractory haemoglobin (f_a), % total haemoglobin			
		3rd day	7th day	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***
1	F	+−	+	1.3	4.0	6.3	0.8	13.4	18.5	14.2	14.7	75.0	67.5	65.0	75.0
2	M	+	−	1.3	4.1	1.6		13.8	20.0	18.9		77.5	78.5	77.0	
3	M	−	−	0.7	2.1	1.1	0.6	15.0	20.0	22.0	19.0	50.0	45.0	44.0	40.0
4	M	++		0.7				17.5	18.0			76.0	77.0		
5	M	−	−		2.2	2.1	(1.5)	15.0	22.3	19.5	(15.6)	82.5	83.0	80.0	(57.0)
6	F	−	−	0.7	3.6	3.3		14.5	18.5	18.1		79.5	77.5	77.5	
7	F	+	−	1.0	7.3	3.9		20.0	21.0	20.3		71.0	61.0	66.0	
8	F	+−	−	1.6	3.4	1.2	(1.8)	13.8	21.0	20.0	(20.0)	69.0	66.0	55.0	(59.0)
9	M	+	+	1.0	5.9	4.3	(0.6)	15.2	16.8	17.7	(17.7)	74.0	68.0	83.0	(65.0)
10	M	−	−		1.1	1.2	(0.7)	15.5	19.5	15.5	(15.5)	82.5	79.5	78.5	(77.5)
11	M	+++	++	2.0	15.2	8.6	(2.8)	14.5	18.2	16.9	(15.2)	80.0	68.5	75.0	(61.5)
12	F	+	++	1.5	5.5	10.0	(1.0)	14.6	20.4	20.5	(18.2)	75.0	76.0	75.0	(69.0)
13	M	++	++	1.5	9.0	9.0		13.0	19.0	20.5		69.0	72.0	77.5	
14	M	−	−	1.1	2.6	2.1		17.0	21.0	19.8		76.5	75.0	74.0	
15	F	+	+	3.5	9.1	6.5		17.5	17.5	17.3		82.5	82.5	83.0	
16	M	++	+	2.2	6.9	5.2		15.2	22.0	18.1		77.5	74.0	76.0	
17	M	+	+	1.8	4.2	4.3		14.7	20.1	21.4		79.5	78.5	78.5	
18	M	+−	−	1.0	3.5	3.0	2.3	15.7	20.5	19.5	10.5	67.0	83.0	85.0	76.0
19	M	+	+	1.6	3.4	3.4			19.6	17.2		69.0	76.0	75.0	
20	M	−	−	1.0	1.7	1.0		14.9	20.7	17.7		72.5	70.0	66.5	

* Degrees of icterus represented by:
− no jaundice.
+− doubtful.
+ slight.
++ moderate.
+++ severe.

** All cases reported were free from jaundice at birth, while of those examined at three or four weeks of age none showed any signs of icterus.

*** Figures in brackets represent values determined at three weeks, the remainder at four weeks.

APPENDIX—*Concluded*

COMPLETE DATA OF CASES SUMMARIZED IN TABLE II—*Concluded*

Case No.	Sex	Degree of icterus *, **		Bilirubin, mgm./100 ml.				Total haemoglobin, gm./100 ml.				Refractory haemoglobin (f_3), % total haemoglobin			
		3rd day	7th day	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***
21	M	+	+	1.6	4.0			14.4	20.7	17.4		67.0	76.0	75.0	
22	M	+	++	2.0	5.4	10.0		11.4	19.8	17.5		81.0	81.0	81.0	
23	M	−	−	0.6	2.5	1.5		13.0	19.4	15.6		80.0	85.0	85.4	
24	F	+	−	1.1	7.7	2.0		12.6	14.6	16.6		87.0	87.0	87.0	
25	M		−	0.9		1.8	2.1	15.0		18.6	16.0	82.5		79.5	79.5
26	M	−	+	2.0		4.1		16.0	18.6	15.0		75.5	77.0	75.5	
27	F	+−	+−	1.1	3.8	4.3		14.3	21.5	18.5		86.0	84.0	83.0	
28	M	++	+	2.3	6.6	5.5	0.9	16.6	16.3	16.0	12.8	85.5	83.0	80.0	77.0
29	F	−	−	1.2	2.9	0.9	0.7	15.7	21.0	16.4	16.7	78.0	78.0	78.5	74.0
30	M	−	−	2.0	4.2	3.0		15.5	17.3	18.0		77.5	79.5	77.0	
31	M	+	++	2.4		6.2		14.1	18.2	15.6		86.5	87.0	83.0	
32	M	+	++	2.1	5.4	7.5		15.1	21.8	22.5		87.0	81.5	81.5	

* Degrees of icterus represented by: − no jaundice.
+− doubtful.
+ slight.
++ moderate.
+++ severe.

** All cases reported were free from jaundice at birth, while of those examined at three or four weeks of age none showed any signs of icterus.

*** Figures in brackets represent values determined at three weeks, the remainder at four weeks.

FURTHER OBSERVATIONS ON INTERFERENCE BETWEEN LYMPHOCYTIC CHORIOMENINGITIS AND MM VIRUSES[1]

By A. J. Rhodes and Marion Chapman

Abstract

Well marked interference is demonstrable when LCM virus is injected cerebrally in hamsters and MM virus peritoneally four or seven days later, the usual paralyzing action of the latter virus being prevented. This interference can still be demonstrated when the MM virus is injected 30 days after the LCM virus, but not when the sequence of the injections is reversed. The unparalyzed survivors of a successful interference experiment are actively immune to LCM virus. The brain, cord, and viscera of survivors, tested 10 and 11 days after the beginning of an interference experiment, contain the same amount of LCM virus as the organs of controls inoculated with this virus alone. The same organs, however, contain significantly less MM virus than the organs of controls inoculated with MM virus only. It appears that in a successful interference experiment, MM virus is prevented from multiplying in the organs of the hamster for at least six or seven days. Observations on the distribution of LCM and MM viruses in the viscera, brain, and cord of normal hamsters show that in both instances the blood is quickly invaded, and thereafter viral growth occurs in the viscera as well as the central nervous system. The reaction between the two viruses probably therefore occurs in viscera as well as central nervous system.

Introduction

We have already drawn attention to the interference phenomenon that can be demonstrated in hamsters inoculated with the antigenically unrelated lymphocytic choriomeningitis (LCM) and MM viruses (7). When the LCM virus is injected cerebrally, and the MM virus peritoneally four to seven days later, a significant number of the animals fails to develop paralysis due to MM virus. This reaction appeared worthy of further study as a model of the type of interference that occurs between unrelated viruses inoculated by different routes. We now report on our study of three aspects of this phenomenon: (1) An investigation of the distribution of the viruses in the organs of normal hamsters. (2) A study of the distribution of the viruses in hamsters surviving an interference experiment. (3) An examination of the relationship between interference and active immunity. The results of these studies will be reported in three sections, and will then be discussed together in the final section.

I. The Distribution of LCM and MM Viruses in Normal Hamsters

The Distribution of LCM Virus in Hamsters Inoculated Cerebrally

This study, and the parallel one with MM virus, was carried out with a view to determining, if possible, the organs that might be involved in interference. Incidental to this study, observations were made on the pathogenesis of these infections in hamsters.

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Methods

The details of preparation of the pools of LCM virus used in the present study were given in our earlier report, and remained the same in this investigation.

A group of hamsters 21 to 24 days old was inoculated cerebrally with 0.05 ml. of a $10^{-1.7}$ dilution of LCM infected mouse brain, a dose previously found to cause recognizable signs of infection in all hamsters of this age. At stated intervals after inoculation, single animals were anesthetized, and 1 to 2 ml. of heart blood withdrawn and treated with heparin. The animal was then perfused through the heart with 50 to 60 ml. of 0.1% heparin in saline. The liver, spleen, kidney, brain, and cord were removed and stored in the dry-ice box. Subsequently, dilutions of whole blood, and suspensions of the solid organs were prepared; serial 10-fold dilutions were inoculated cerebrally into groups of six 12 to 14 gm. mice in 0.03 ml. amounts, in order to establish the approximate virus content of the various organs. Deaths of mice were recorded and the LD_{50} levels were determined by the method of Reed and Muench customarily used in this type of work.

Results

As can be seen from Table I, LCM virus was first demonstrated in the blood 24 hr. after cerebral inoculation and viraemia continued throughout the 12 day period of the experiment.

TABLE I

TITRATION OF HAMSTER ORGANS HARVESTED AT INTERVALS AFTER CEREBRAL INOCULATION OF LCM VIRUS

Interval after inoculation	Titer of virus in terms of LD_{50} for mice		
	Blood	Viscera	Brain and cord
Hours			
2	Nil		
6	Nil	$10^{-1.0}$	$10^{-1.0}$
12	Nil		$10^{-1.5}$
24	$10^{-1.0}$	$10^{-2.7}$	$10^{-1.4}$
36	$10^{-2.5}$		$10^{-4.5}$
48	$>10^{-3.0}$*	$10^{-4.0}$	$10^{-6.0}$
60	$10^{-3.2}$		$>10^{-5.5}$*
72	$>10^{-5.0}$*		$>10^{-6.0}$*
84	$>10^{-5.0}$*	$>10^{-5.0}$*	$>10^{-6.0}$*
96	$10^{-4.2}$	$>10^{-6.0}$*	$>10^{-6.0}$*
Days			
5			$10^{-6.0}$
6	$10^{-5.4}$	$10^{-6.3}$	$10^{-5.7}$
7	$>10^{-5.0}$*		$10^{-5.5}$
8	$10^{-5.4}$	$>10^{-6.5}$*	$10^{-5.4}$
9			$>10^{-5.0}$*
10	$10^{-5.0}$	$10^{-6.3}$	$10^{-5.4}$
11			$>10^{-5.0}$*
12	$10^{-5.3}$	$10^{-5.7}$	$10^{-5.2}$

* *End points were not reached in these titrations, owing to the unexpectedly large amounts of virus present.*

Virus was recovered from the pooled viscera as early as six hours, presumably having been transported there from the site of inoculation by the blood stream, although at this early stage no virus was actually recovered from the blood. Large quantities of virus persisted for at least 12 days.

In the brain and cord, viral multiplication began between 24 and 36 hr. after inoculation, maximal quantities of virus being found between two and five days after inoculation; large amounts were still present 12 days after inoculation.

The Distribution of MM Virus in Hamsters Inoculated Peritoneally

Methods

MM virus pools were prepared as previously described. A group of hamsters 21–24 days old was inoculated peritoneally with 0.5 ml. of a 10^{-2} dilution of infected mouse brain, a dose known to paralyze practically all animals of this age. At frequent intervals after inoculation, hamsters were sacrificed, and the organs removed following the procedure used for the animals infected with LCM virus.

Results

The results are given in Table II, from which it will be seen that MM virus rapidly entered the blood, being demonstrable as early as four hours. Large amounts of virus circulated from 20 to 36 hr. after inoculation and throughout the duration of the experiment.

TABLE II

TITRATION OF HAMSTER ORGANS HARVESTED AT INTERVALS AFTER PERITONEAL INOCULATION OF MM VIRUS

Interval after inoculation, hr.	Titer of virus in terms of LD_{50} for mice		
	Blood	Viscera	Brain and cord
2	Nil		
4	$10^{-2.5}$	$10^{-3.2}$	
6		$10^{-4.0}$	
8	$10^{-3.0}$		
12	$10^{-3.2}$	$10^{-4.4}$	$10^{-1.7}$
16	$10^{-3.0}$		$10^{-4.3}$
20	$>10^{-5.0*}$		$10^{-4.4}$
24	$>10^{-5.0*}$	$10^{-4.4}$	$10^{-5.3}$
30		$10^{-4.0}$	
36	$>10^{-5.0*}$	$10^{-4.8}$	$10^{-6.4}$
42	$10^{-4.2}$	$10^{-4.5}$	$10^{-6.2}$
48	$10^{-3.5}$	$10^{-3.7}$	$10^{-5.6}$
54	$10^{-4.1}$		$10^{-5.5}$
60	$10^{-4.2}$	$10^{-4.7}$	$10^{-6.0}$
72	$10^{-4.5}$	$10^{-4.0}$	$10^{-6.0}$

**End points were not reached in these titrations, owing to the unexpectedly large amounts of virus present.*

In the pooled viscera, the amount of virus remained fairly high and relatively constant from shortly after inoculation till the conclusion of the experiment.

In the brain and cord, only a small amount of virus was present at 12 hr. after peritoneal inoculation. Between 16 and 36 hr., there was a marked increase in the amount of virus, which remained relatively constant till the end of the experiment.

II. The Distribution of LCM and MM Viruses in Survivors of Interference Experiments

Methods

This study was carried out using the survivors of an interference experiment referred to in our previous report as Experiment 4; the interval between the inoculation of LCM and MM viruses was four days, and 9/10 animals failed to develop paralysis, whereas only 2/10 controls failed to become paralyzed.

Two unparalyzed hamsters were sacrificed 10 days after the start of the experiment, and two 11 days after the start, that is to say respectively six and seven days after the inoculation of MM virus. Appropriate controls, inoculated with LCM or MM viruses only were likewise sacrificed. The brain, cord, and viscera were removed and stored frozen. Later, suspensions of these organs were prepared in broth, serial dilutions made, and inoculated cerebrally in groups of six to eight mice (0.03 ml. amounts). In no case were less than four serial 10-fold dilutions tested. The animals were closely examined, and the day of death recorded. Death occurring within five days was regarded as caused by MM virus, whereas sickness developing from the sixth day on was attributed to LCM virus. This method, of course, demonstrated the presence of MM virus rather than LCM virus, for mice inoculated with a mixture of these viruses died from the short incubation MM illness rather than the more slowly progressive LCM infection. However, as the MM virus content of the organs of survivors was very low, it is believed that the method also estimated reasonably accurately the amount of LCM virus in these organ suspensions.

Reference to Table III, which gives details of the time of death of the various mice inoculated in this experiment, shows that when serial dilutions of organs known to contain MM virus alone were injected, nearly 90% of the mice died by the end of the fifth day. In the same five day period only about 3% of mice injected with suspensions of organs containing LCM virus died. It appears, therefore, that our criteria for apportioning the cause of death in the mice inoculated with organ suspensions which might contain both viruses were acceptable.

Results

The concentrations of the viruses in the organs of hamsters sacrificed 10 days after the start of the experiment are given in Table IV. It will be seen that

TABLE III

RESULT OF INOCULATION OF MICE WITH DILUTIONS OF INFECTED HAMSTER ORGANS

	Number of mice dying	Number of mice dying by end of fifth day	Deaths beyond five days
Organs from hamsters receiving MM virus only	201	173 (86.6%)	28 (13.4%)
Organs from hamsters receiving LCM virus only	356	12 (3.4%)	344 (96.6%)
Organs from hamsters inoculated with both viruses	366	14 (3.8%)	352 (96.2%)

TABLE IV

DISTRIBUTION OF MM AND LCM VIRUSES IN ORGANS OF HAMSTERS SACRIFICED 10 DAYS AFTER BEGINNING OF EXPERIMENT

Hamster No.	State as regards inoculation	Approximate content of LCM virus in:			Approximate content of MM virus in:		
		Viscera	Brain	Cord	Viscera	Brain	Cord
1	Controls: MM virus only				$10^{-2.3}$	$10^{-4.2}$	$10^{-3.5}$
2					$10^{-2.2}$	$10^{-4.5}$	$10^{-4.0}$
3	Controls: LCM virus only	$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$			
4		$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$			
5	Survivors of interference experiment	$10^{-3.2}$	$>10^{-5.5}$	$>10^{-5.5}$	$<10^{-1.0*}$	$<10^{-1.0*}$	$<10^{-1.0*}$
6		$10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$	$10^{-1.3}$	$<10^{-1.0*}$	$<10^{-1.0*}$

* *Indicates no virus detected in 10^{-1} dilution of organs.*

considerable quantities of MM virus were found in the organs of controls. By comparison, no MM virus was recovered from 1:10 suspensions of the brain or cord of the survivors of the interference experiment, and was found in small amount in the viscera of one animal only. LCM virus was found in essentially the same quantities in the organs of survivors and controls.

The concentrations of the viruses in the organs of hamsters sacrificed a day later, i.e. 11 days after the beginning, were as shown in Table V. These results are in agreement with those given in Table IV, the content of MM virus being definitely less in the survivors of the interference experiment than in the controls inoculated with MM only.

TABLE V

DISTRIBUTION OF MM AND LCM VIRUSES IN ORGANS OF HAMSTERS SACRIFICED 11 DAYS AFTER BEGINNING OF EXPERIMENT

Hamster No.	State as regards inoculation	Approximate content of LCM virus in:			Approximate content of MM virus in:		
		Viscera	Brain	Cord	Viscera	Brain	Cord
1	Controls: MM virus only				$10^{-1.7}$	$10^{-2.4}$	$10^{-2.8}$
2					$10^{-1.5}$	$10^{-2.6}$	$10^{-3.1}$
3					$10^{-1.6}$	$10^{-2.6}$	$10^{-2.4}$
4					$10^{-1.8}$	$10^{-3.6}$	$10^{-4.0}$
5	Controls: LCM virus only	$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$			
6		$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$			
7	Survivors of interference experiment	$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$	$<10^{-1.0}$*	$<10^{-1.0}$*	$<10^{-2.0}$**
8		$>10^{-3.5}$	$>10^{-5.5}$	$>10^{-5.5}$	$<10^{-1.0}$*	$<10^{-1.0}$*	$<10^{-2.0}$**

* *Indicates no virus detected in 10^{-1} dilution of organs.*

** *Indicates no virus detected in 10^{-2} dilution, which was the most concentrated tested.*

III. Relationship Between Interference and Active Immunity

Susceptibility of New Strain of Hamster

It became necessary at this stage to change the source of supply of hamster from a local breeder to Tumblebrook Farms, and accordingly the susceptibility to MM virus of the two strains of golden hamster was compared. As shown in Table VI, young Tumblebrook hamsters, three to four weeks old, proved

TABLE VI

SUSCEPTIBILITY OF TWO STRAINS OF GOLDEN HAMSTER TO MM VIRUS

Source of hamster (aged 3–4 weeks)	Incidence of paralysis following intraperitoneal inoculation of dilutions of MM virus	
	10^{-2}	10^{-3}
Local	25/33 (76%)	22/33 (66%)
Tumblebrook Farms	36/38 (95%)	17/18 (94%)

somewhat more susceptible to MM virus than our local hamsters. Several satisfactory interference experiments have been conducted with the new strain which is eminently suitable for this work.

Interference Experiment

In order to have available for study a number of survivors, a further interference experiment was carried out along the usual lines but using the Tumblebrook Farms hamster. The results are shown in Table VII, from which it will

TABLE VII

DETAILS OF INTERFERENCE EXPERIMENT IN HAMSTERS

Interval between primary injection of LCM and inoculation of MM virus	Dilution of LCM virus in form of mouse brain (0.05 ml. amounts)	Dilution of MM virus in form of mouse brain (0.5 ml. amounts)	Numbers of animals that developed paralysis on inoculation with MM	
			Treated with LCM	Controls, untreated with LCM
4 days	10^{-2}	10^{-2}	1/30*	18/20*

* *These differences are highly significant.*

be evident that of 30 animals receiving both viruses, only one became paralyzed so that 29 survivors were available.

Cross-Resistance Tests

These 29 survivors were held for 30 days, and were then divided into groups and challenged by the cerebral route with either LCM or MM virus (0.05 ml. of a 10^{-1} suspension). Appropriate control groups of animals of the same age at the time of challenge, but previously uninoculated with either virus, were injected at the same time. The results of challenge with MM virus are given in Table VIII and with LCM virus in Table IX.

TABLE VIII

RESULTS OF CEREBRAL CHALLENGE WITH MM VIRUS IN HAMSTERS SURVIVING INTERFERENCE EXPERIMENT

Description of animals challenged	Number of animals challenged*	Results of challenge
Normal controls of same age as survivors	10	10/10 became paralyzed
Inoculated with MM only at time of interference experiment; convalescent from paralysis	9	None showed any extension of paralysis
Unparalyzed survivors of interference experiment	14	None became paralyzed
Inoculated with LCM only at time of interference experiment	6	Only 1/6 developed transient paralysis

* *Challenge carried out 30 days from start of original interference experiment.*

From Table VIII it will be seen that when MM virus was given cerebrally to normal controls of the same age as the survivors, all became paralyzed. The animals inoculated with MM only at the time of the interference experiment and convalescent from their paralytic attacks, showed no extension of

TABLE IX

RESULTS OF CEREBRAL CHALLENGE WITH LCM VIRUS IN HAMSTERS SURVIVING INTERFERENCE EXPERIMENT

Description of animals challenged	Number of animals challenged*	Results of challenge
Normal controls of same age as survivors	10	Only 3/10 became sick.
Inoculated with LCM only at time of interference experiment	6	1 died within 7 days; remainder healthy
Inoculated with MM only at time of interference experiment; convalescent from paralysis	9	1 died within 7 days; 4 showed signs of mild LCM infection; 4 remained *in statu quo*
Unparalyzed survivors of interference experiment	13	None developed any sickness attributable to the challenge

* *Challenge carried out 30 days from start of original interference experiment.*

paralysis on challenge and had presumably become actively immune. None of 14 survivors of the interference experiment developed paralysis when challenged with MM. This might be interpreted as due to active immunity, but consideration must be given to the fact that of six animals inoculated with LCM virus alone at the time of the interference experiment only one became paralyzed when challenged with MM 30 days later. It would appear therefore that the blocking action of LCM against MM virus persists for at least 30 days.

The interpretation of the results shown in Table IX is rendered difficult by the fact that the preparation of LCM virus, although fully potent for the young animals used at the start of the experiment, was of reduced virulence for the animals 30 days older, only 3/10 of which were affected clinically. Nevertheless, it would appear that the 13 survivors of the interference experiment were actively immune to challenge with LCM virus, as they developed no further sickness. It is of interest to note that the nine animals originally inoculated with MM virus alone showed no resistance to the LCM challenge. There does not therefore appear to be any interference demonstrable over the 30 day period when the MM virus is given first.

IV. Discussion

The problem of interference between animal viruses has been much studied of recent years, and Findlay (4) and Henle (6) have both recently reviewed the question. In our study of the problem we have investigated the blocking action of lymphocytic choriomeningitis (LCM) virus injected cerebrally in hamsters against the MM virus injected peritoneally, as first described by Dalldorf and Whitney (3). This interference is evidenced by the sparing from MM paralysis noted in these animals previously inoculated with LCM virus.

A similar sparing effect from clinical signs of infection or from death has been demonstrated with several other pairs of antigenically unrelated animal viruses. For example, when Rift Valley fever virus is inoculated intraperitoneally in rhesus monkeys, some of the animals are protected against the effects of an inoculation of yellow fever virus given shortly afterwards (5). To give another example, mice injected cerebrally with influenza virus withstand an injection by the same route of the unrelated Western equine encephalomyelitis virus (9). In this type of interference, it is usual for the interfering (blocking) virus to cause a slowly progressive infection which by itself proves lethal to only a small percentage of animals. The blocked virus on the contrary is one that causes a rapidly progressive, commonly fatal infection in control animals.

Of particular interest in the study of interference between such viruses is the actual fate of the virus which appears to be blocked. Some such investigations have been made. For example, Dalldorf (2) studied the interference that can be demonstrated in monkeys injected cerebrally with LCM and the MV strain of human poliomyelitis virus; he found that the sparing effect was associated with an absence of an infectious amount of MV virus in the cervical enlargement of the spinal cord. Similar observations were made by Vilches and Hirst, the growth of Western equine encephalomyelitis virus being completely suppressed by influenza virus (9). A different site of interaction was studied by Andrewes (1) who injected mixtures of virus III and fibroma viruses intradermally in rabbits. Virus III suppressed completely the development of fibromata, and the rabbits did not become resistant to fibroma virus. These three observations concern interference between unrelated viruses injected into an animal by the same route. In the reaction that we have studied, the situation is more complicated because the viruses are inoculated by different routes, the MM virus reaching the central nervous system presumably by the blood stream.

In studies on the distribution of LCM and MM viruses in normal hamsters we found that after cerebral inoculation, LCM virus rapidly invades the rest of the body, being found in the blood and viscera before it starts to multiply in the brain and cord. At the critical period of four to seven days after inoculation, when MM virus is inoculated in interference experiments, large amounts of LCM virus are present in the brain, cord, viscera, and blood. Following peritoneal inoculation, MM virus can be recovered from the blood and viscera after four hours. By 16 hr., multiplication is proceeding in the brain and cord. Virus can be recovered in large amount from blood, viscera, and central nervous system for at least 72 hr.

It is evident, therefore, that both LCM and MM viruses proliferate in the viscera as well as the central nervous system of hamsters, and they can be regarded as showing both viscero- and neurotropic properties. Incidentally, it is of interest to note that large amounts of either virus can be recovered from organs substantially before there are any recognizable signs of sickness.

In studies on the distribution of LCM and MM viruses in survivors of interference experiments it was shown that LCM virus proliferates in the viscera, brain, and cord to the same extent as in controls. The state of affairs with MM virus was, however, found to be markedly different, for the virus was not detected at all in the brain or cord of three of the four animals tested and only in low concentration in the viscera of one of the animals. More extensive tests, which we are not at the moment in a position to perform, should be carried out to determine whether this initial suppression of MM virus growth continues in animals sacrificed at a later stage of the experiment. One would expect the same conditions to prevail, as clinical observation of surviving hamsters over a four week period has shown no tendency to the development of MM paralysis after the sixth or seventh day following the inoculation of this virus. Our results are therefore in agreement with those of workers using other pairs of viruses who demonstrated by infectivity tests that growth of the blocked virus was in fact markedly inhibited. Our studies bring forward the additional observation that inhibition of growth occurs not only in the brain and cord but also in the viscera. It is probable that a very considerable number of host cells must be altered by the LCM virus in some way that prevents multiplication of the MM virus, during the critical period after inoculation of the second (MM) virus. It would seem reasonable to suggest that the reason why interference cannot be demonstrated when the LCM and MM viruses are injected 48 hr. apart (7) is that at that early stage not enough multiplication of LCM virus has occurred to alter the susceptibility of the cells available to the MM virus.

Another aspect of the interference phenomenon that interested us was relationship to immunity. We found that unparalyzed survivors do not develop any sickness attributable to a challenge of LCM virus given 30 days after the beginning of the experiment, so that active immunity to the blocking virus probably develops, as one might expect from the fact that this virus is uninhibited in its growth. Survivors also resist a challenge of MM virus given 30 days after the beginning of the experiment. However, one cannot state definitely that this resistance is due to the development of active immunity, because animals inoculated in the first instance with LCM virus alone are likewise resistant to MM virus 30 days later. The probable explanation is that LCM virus survives in the brain and probably viscera for this long period after direct cerebral inoculation, and that interference is associated with the continued presence of active LCM virus.

The finding that interference can still occur after 30 days is of considerable general interest, because a not uncommon method of investigating the antigenic relationship of two viruses is to challenge immunized animals. Before suggesting, therefore, that cross-resistance between two viruses implies a common antigenic structure, it is necessary to exclude the possibility that resistance is due to interference set up by virus persisting from the original immunizing inoculations. In this connection, one may recall the observations of Schlesinger, Olitsky, and Morgan (8) relating to the same type of problem. These workers

found that guinea pigs rendered resistant to Western equine encephalomyelitis virus by vaccination and by cerebral injection resisted cerebral injection of the antigenically distinct Eastern equine encephalomyelitis and vesicular stomatitis viruses.

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